

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
31 July 2003 (31.07.2003)

PCT

(10) International Publication Number  
**WO 03/061580 A2**

- (51) International Patent Classification<sup>7</sup>: **A61K** (72) Inventors; and  
(21) International Application Number: PCT/US03/01622 (75) Inventors/Applicants (for US only): PAVAN, William, J.  
(22) International Filing Date: 17 January 2003 (17.01.2003) [US/US]; 7724 Warbler Lane, Derwood, MD 20855 (US).  
(25) Filing Language: English LOFTUS, Stacie, K. [US/US]; 10829 Monticello Drive,  
(26) Publication Language: English Great Falls, VA 22066 (US).  
(30) Priority Data: 18 January 2002 (18.01.2002) US (74) Agents: SAMPSON, Elizabeth, R. et al.; Townsend and  
60/349,929 Townsend and Crew LLP, Two Embarcadero Center, Eighth  
(71) Applicant (for all designated States except US): THE Floor, San Francisco, CA 94111 (US).  
GOVERNMENT OF THE USA as represented by  
THE SECRETARY OF THE DEPT. OF HEALTH  
AND HUMAN SERVICES [US/US]; OTT-NH, 6011  
Executive Boulevard, Room 325, Rockville, MD 20852  
(US).  
(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE,  
SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ,  
VC, VN, YU, ZA, ZM, ZW.

[Continued on next page]

(54) Title: ALTERATION OF RAB38 FUNCTION TO MODULATE MAMMALIAN PIGMENTATION



b

|        |   |       |                                    |
|--------|---|-------|------------------------------------|
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |
| HRAB38 | 1 | ----- | MTAPKHEKLYKLLVIGDLGVGKTSIIKRYVHONF |

(57) Abstract: The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

BEST AVAILABLE COPY

WO 03/061580 A2



(84) **Designated States (regional):** ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## ALTERATION OF RAB38 FUNCTION TO MODULATE MAMMALIAN PIGMENTATION

### CROSS-REFERENCES TO RELATED APPLICATIONS

- 5 [0001] This application claims priority from U.S. Provisional Patent Application No. 60/349,929 filed January 18, 2002, and incorporates the contents thereof.

### STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

- 10 [0002] NOT APPLICABLE

### REFERENCE TO A "SEQUENCE LISTING," A TABLE, OR A COMPUTER PROGRAM LISTING APPENDIX SUBMITTED ON A COMPACT DISK.

- [0003] NOT APPLICABLE

15

### FIELD OF THE INVENTION

- [0004] The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.
- 20

### BACKGROUND OF THE INVENTION

- [0005] Melanocytes are specialized pigment producing cells that are responsible for coloration of skin, eyes and hair. Coat color alterations resulting from melanocyte defects are easily identifiable in mice. These mouse mutants are proving valuable for the identification of candidate human disease genes and for the elucidation of mechanisms underlying cellular function. To date, there are approximately 100 loci in the mouse that, when mutated, affect pigmentation. However, the underlying genetic defect has not been identified in about 60 of these loci (*See*, The Jackson Laboratory's Mouse Genome Informatics web site).
- 25
- [0006] Disorders with reduced pigmentation can be placed into two groups according to whether they affect melanocyte differentiation or whether they affect the function of the pigment producing organelle in the melanocyte, the melanosome. Examples of the first group
- 30

include Piebaldism and Waardenburg Syndrome, characterized by a localized absence of melanocytes resulting in "white patch" patterns. Genes affected in these disorders, *KIT*, *MITF*, *PAX3*, *SOX10*, *EDNRB*, *EDN3*, are involved in specification, migration and survival of the melanocyte lineage (Jackson, Hum Mol Genet 6:1613-1624 [1997]). Mouse models of these disorders have characteristic spotted coat patterns (Jackson, Hum Mol Genet 6:1613-1624 [1997]). Oculocutaneous albinism (OCA) I-IV, Chediak-Higashi Syndrome (CHS), Hermansky-Pudlak Syndrome (HPS) I-III and Griscelli syndrome (GS) correspond to the second group. The molecular defects contributing to the reduced pigmentation in OCA occur in genes (*TYR*, *TYRP1*, *P* and *AIM1*) that mainly effect melanosome formation and the amount and type of melanin pigment formed (King *et al.*, in Scriver *et al.*, (eds.) The metabolic basis of inherited disease, 7th ed. (McGraw Hill, New York) gyp. 4353-4392 [1995]); and Newton *et al.*, Am J Hum Genet 69:981-988 [2001]). Genes responsible for HPS, CHS and GS are involved in the regulation of vesicle traffic including melanosomes within the cell and include *HPS1*, *AP3*, *HPS3*, *CHSI*, *MYO5A* and *RAB27A* (Jackson, Hum Mol Genet 6:1613-1624 [1997]; and Marks and Seabra, Nat Rev Mol Cell Biol 2:738-748 [2001]).

[0007] Although many genes have been associated with pigmentation disorders, in view of the genetic heterogeneity of these disorders in both mice and humans, there is a need in the art to identify additional candidate disease genes in these and other species.

#### BRIEF SUMMARY OF THE INVENTION

[0008] The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

[0009] The present invention provides an isolated nucleic acid that comprises a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO: 11, and SEQ ID NO:12. In some embodiments, the nucleic acid is deoxyribonucleic acid. In other embodiments, the nucleic acid is the complement of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12. In related embodiments, a vector comprising the nucleic acid is provided. Also provided is a host cell comprising the vector. Additionally, the present invention provides a protein encoded by the nucleic acid sequence set forth in SEQ ID NO:9.

[0010] In some embodiments, the present invention provides methods for detecting mutations in *Rab38* comprising the steps of: amplifying at least a portion of *Rab38* from

genomic DNA to yield a *Rab38* amplification product; purifying the *Rab38* amplification product; and sequencing the *Rab38* amplification product. In preferred embodiments, the amplifying is accomplished using a polymerase chain reaction. In related embodiments, the at least a portion of *Rab38* genomic DNA is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence. In some particularly preferred embodiments, the at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3. In some embodiments, the genomic DNA is mammalian genomic DNA. Also provided are embodiments where the purifying is accomplished via size selection.

[0011] The present invention further provides methods for detecting mutations in *Rab38* comprising the steps of amplifying at least a portion of *Rab38* from genomic DNA to yield a *Rab38* amplification product; digesting the *Rab38* amplification product to yield a digested *Rab38* amplification product; and electrophoresing the digested *Rab38* amplification product. In preferred embodiments, the amplifying is accomplished using a polymerase chain reaction.

In some embodiments, the at least a portion of *Rab38* genomic DNA is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence. In preferred embodiments, the at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3. In particularly preferred embodiments, the genomic DNA is mammalian genomic DNA.

[0012] The present invention also provides methods for screening for biologically active agents to modulate RAB38 activity, comprising the steps of providing: melanocytes comprising RAB38 activity, and a candidate agent; and exposing the melanocytes to the candidate agent to yield treated melanocytes; and measuring the modulation of the RAB38 activity of the treated melanocytes by the candidate agent. In some embodiments, the RAB38 activity comprises GTPase activity. In some related embodiments, the RAB38 activity comprises GTP binding activity or GDP release. In some preferred embodiments, the RAB38 activity comprises TYRP1 trafficking to melanosomes or RAB38 trafficking to melanosomes.

[0013] In some embodiments, the present invention provides kits for screening for biologically active agents that modulate RAB38 activity, comprising: plurality of melanocytes comprising RAB38 activity, wherein the melanocytes are provided within a container, and instructions for determination of RAB38 activity in the melanocytes. In some preferred embodiments, kits further comprise the means to analyze RAB38 activity. In some

related embodiments, the means to analyze RAB38 activity comprises an assay to assess GTPase activity, an assay to assess GTP binding activity, an assay to assess GDP release, an assay to assess TYRP 1 trafficking to melanosomes, or an assay to assess RAB38 trafficking to melanosomes.

5 [0014] The present invention also provides kits for the detection of mutations in *RAB38* comprising at least two primer sequences suitable for amplification of at least a portion of *RAB38*, and instructions for utilizing the kit. In some preferred embodiments, the primer sequences are selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22. In some related embodiments, the kit is suitable for use in the polymerase chain reaction. The invention also provides  
10 embodiments further comprising reagents for digesting nucleic acid.

[0015] In some embodiments, the present invention also provides kits for diagnosing defects in melanosome function, comprising melanocytes comprising *RAB38* and instructions for assessing defects in melanosome function. In some preferred embodiments, the kits  
15 further comprise the means to analyze RAB38 activity. In some related embodiments, the means to analyze RAB38 activity comprises an assay to assess GTPase activity, an assay to assess GTP binding activity, an assay to assess GDP release, an assay to assess TYRP 1 trafficking to melanosomes or an assay to assess RAB38 trafficking to melanosomes.

## 20 BRIEF DESCRIPTION OF THE DRAWINGS

[0016] Figure 1 depicts a microarray expression profile of various pigmentation control genes and *Rab38*. Cluster analysis identified nine genes known to be involved in pigmentation. Columns to the right list mouse and human pigmentation disorders corresponding to a given gene. *Rab38* (arrow) was found within the same expression profile  
25 cluster as the nine control genes known to be involved in pigmentation. Hybridized sample cell lines are listed across the top of the expression profile. Relative gene expression is evaluated as a calibrated ratio (sample cell line / MnSOD6c11 reference). Pseudocolor scale for ratio values is shown below.

[0017] Figure 2 illustrates that RAB38, like known melanogenic enzymes, is expressed in  
30 the retinal pigmented epithelium (RPE). All panels show the eye at embryonic day E11.5. The melanogenic enzymes expressed in the RPE at this developmental stage include tyrosinase (*Tyr*; Panel A), tyrosinase related protein 1 (*Tyrl*; Panel B), and dopachrome tautomerase (*DCT/TTyrp2*; Panel C). Additional control genes from the microarray cluster data that are expressed in the RPE include melastatin1 (*Mln*; Panel D) and *Aiml/Matp* (Panel

E). *Rab38* is expressed in the RPE at E11.5 (Panel F) and at E10.5 and E12.5. The scale bar equals 100 pm.

[0018] Figure 3 illustrates the *Rab38* map location and the phenotype of the chocolate (*cht/cht*) mouse. Panel A provides a map of human chromosome 11 showing that human *Rab38* maps 1.4 Mb distal to tyrosinase and 2.5 cM proximal to EED genes. In the corresponding mouse chromosome 7, syntenic region gene order is conserved (e.g., the *cht* locus maps to the same interval as *Rab38*). Panel B provides a photograph of C57B1/6J +/+ (left side, black) and C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* (right side, brown) mice. Panel C shows the eyes from 2 day old wildtype C57B1/6J +/+ mice with normal pigmentation, while Panel D shows that the eyes from *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* mice exhibit much less pigmentation.

[0019] Figure 4 indicates that the *Rab38* mutation causes the chocolate (*cht*) mouse phenotype. Panel A provides a comparison of *Rab38* sequence between wildtype C57B1/6J +/+ DNA (SEQ ID NO: 1) and mutant C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* DNA (SEQ ID NO:2), revealing a G146T nucleotide change (arrow) in the *cht* allele. This nucleotide change was never seen in eight additional inbred strains analyzed. Panel B illustrates that the G146T mutation creates a *SexA1* restriction enzyme site in C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* DNA and ablates a *BsaJI* restriction site present in wildtype *Rab38* sequence. A 216 bp region surrounding the G146T nucleotide mutation was amplified from both C57B1/6J +/+ DNA and C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* DNA. *SexA1* digests the PCR fragment of C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* (lane 1), but not C57B1/6J +/+ (lane 2); *BsaJI* digests the PCR fragment of C57B1/6J +/+ (lane 4), but not C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* (lane 3).

[0020] Figure 5 shows that RAB38 G19 is located in the GTP binding pocket. Panel A provides the 3 dimensional location of the amino acid G19 of RAB38 in relation to the nucleotide binding site, as determined using the molecular modeling database (MMDB; Wang *et al.*, Nucleic Acids Res 28:243-245 [2000]), based upon the crystal structure for RAB3a (MMDB 10125; and Dumas *et al.*, Structure Fold Des 7:413-423 [1999]). Overlaying the RAB38 sequence with that of RAB3a identified amino acid S32 of RAB3a as being equivalent to G19 of RAB38. The program Cn3D 3.0 was used to indicate the location of the RAB38 G19 (white), predicting interaction with the bound nucleotide. Protein structure is indicated by color: green,  $\alpha$ - helices; gold,  $\beta$  sheet; blue, random coils; white, site of RAB3a S32 equivalent to RAB38 G19 located at the nucleotide binding site; grey, Mg<sup>++</sup> ion; red grey, GppNHp nucleotide analog. Panel B provides alignments of the highly conserved N-terminal region including human RAB38 (NP\_071732; and SEQ ID NO:3), rat RAB38 (AAA42000; and SEQ ID NO:4), and mouse RAB38 (AK009296.1; and SEQ ID NO:5)

amino acid sequences; and human RAB3a (P20336; and SEQ ID NO:6), human RABS (F34323; and SEQ ID NO:7) and human N RAS (TVHURA; and SEQ ID NO:8) amino acid sequences. Sequence alignment was done using the ClustalW algorithm (Smith *et al.*, Genome Res 6:454-462 [1996]). Bars indicate highly conserved regions that occupy the nucleotide binding pocket, observed in the X ray crystal structure of RAB3a (Ostermeier and Brunger, Cell 96:363-374 [1999]). Black denotes sequence identity, grey denotes sequence conservation, and red denotes the conserved amino acid that is mutated in the chocolate mice.

[0021] Figure 6 illustrates that *cht/cht* melanosomes are similar in morphology to *Tyrpl<sup>b</sup>* melanosomes. Bright field images of melanosomes are shown from the periphery of primary cultured melanocytes, isolated from C57B16/J +/+ mice in Panel A, and from C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* mice in Panel B. Melanosomes from wildtype melanocytes are oval and darkly pigmented, while those from C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* melanocytes are smaller, more circular and less pigmented, resembling melanosomes from *Tyrpl<sup>b</sup>/Tyrpl<sup>b</sup>*, melan b cells. The scale bar equals 2  $\mu$ m.

[0022] Figure 7 shows that RAB38 is a melanosomal protein needed for appropriate TYRP1 trafficking. Bright field and matching confocal images of identical exposure of melanosomes in the periphery of primary melanocytes cultures are provided in the upper and lower panels respectively. Panels A and B provide images of C57B1/6J +/+ melanosomes, Panels C and D provide images of C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* melanosomes, and Panels E and F provide images of melanosomes from melan-a cells transfected with a GFP RAB38 expression construct. TYRP1 distribution was revealed by MEL5 staining in Panels B and D, while GFP RAB38 immunofluorescence shown in Panel F, demonstrates co localization of the GFP RAB38 signal with the highly pigmented, end stage melanosomes. The scale bar for Panels A and D equals 1.6  $\mu$ M, while that for Panels E and F equals 2.4  $\mu$ M.

#### DETAILED DESCRIPTION OF THE INVENTION

[0023] The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation. In addition the method can be used to diagnose and/or treat conditions associated with disorders in pigmentation.

[0024] In order to meet a need in the art for identification of additional pigmentation disease genes, expression profile analysis using cDNA microarrays was conducted done to develop the present invention. Expression profile analysis was utilized for the development of the present invention, as it is a powerful tool for organizing the common patterns found



among thousands of gene expression measurements, and for identifying genes with similar distinctive expression patterns among multiple experimental samples (Eisen *et al.*, Proc Natl Acad Sci USA 95:14863-14868 [1998]). Analysis of genes contained within a cluster has revealed that these genes are often functionally related within the cell (Eisen and Brown, Methods Enzymol 303:179-205 [1999]; and Mody *et al.*, Proc Natl Acad Sci USA 98:8862-8867 [2001]). As detailed below, by using this approach *Rab38* was identified as a candidate pigmentation gene. Further analysis confirmed that *RAB38* is a melanosomal protein, mutated in the mouse pigmentation mutant, *chocolate* (*cht*), and important for the sorting of the melanosomal protein TYRP 1 in melanocytes. These experiments conducted during the development of the present invention provide the first successful use of microarray expression profiling to identify a mammalian pigmentation disease gene.

#### **Expression Profile Analysis Identifies RAB38 as a *chocolate* Candidate Gene**

[0025] In order to identify novel and uncharacterized genes involved in melanocyte function and disease, a collection of cDNA clones to be used for expression profile and functional analyses were generated (Loftus *et al.*, Proc Natl Acad Sci USA 96:9277-9280 [1999]). cDNA clones from IMAGE consortium library 2NbHM (*See*, The National Center for Biotechnology Information's web site for UniGene Library No. 198) were previously shown to be appropriate for gene expression studies aimed at understanding melanocyte development and function (Loftus *et al.*, Proc Natl Acad Sci USA 96:9277-9280 [1999]; and Loftus and Pavan, Pigment Cell Res 13:141-146 [2000]). For this analysis, 4356 cDNA clones from library 2NbHM were printed to glass slides. A total of 17 cell lines representing neural crest and non-neural crest derived tissues were used in this analysis. Included were eleven melanoma cell lines (Bittner *et al.*, Nature 406:536-540 [2000]), three rhabdomyosarcoma cell lines, one glioblastoma cell line, HeLa cells, and 293T cells. Array hybridizations for each of these cell lines were performed in a pair wise fashion, using RNA from cell line MnSOD6 c11 as a reference. MnSOD6 c11 is an amelanotic melanoma cell line rendered non-tumorigenic by the introduction of a region of human chromosome 6 (Trent *et al.*, Science 247:568-571 [1990]). MnSOD6 c11 has been used previously for expression profile analysis of melanoma lines using a different set of cDNA clones (Bittner *et al.*, Nature 406:536-540 [2000]).

[0026] Hierarchical cluster analysis found nine genes (*DCT*, *TYRP1*, *PMEL17*, *AIM-1*, *MELAN A/MART1*, *MLSN*, *ATRN*, *PAX3* and *CHSI*), previously known to be involved in melanocyte function, to cluster together (*See*, Figure 1). On closer analysis of the hierarchical

clustering data an additional gene, *Rab38*, was found to have a similar expression variation to these melanocyte genes (See, Figure 1). Four of the nine melanocyte genes examined (*TYRP1*, *DCT*, *MLSN* and *AIM1*) were expressed in the melanocytes of the retinal pigment epithelium (RPE) at E11.5 (See, Figure 2). Consistent with the placement of *Rab38* within this cluster of genes, whole mount *in situ* analysis demonstrated that *Rab38* was also expressed in the melanocytes of the RPE at this age (See, Figure 2). Northern blot analyses revealed that *Rab38* expression was restricted to the melanocyte derived cell lines (Jager *et al.*, Cancer Res 60:3584-3591 [2000]).

[0027] Utilizing the recently available human genome sequence, *Rab38* was determined to be located on human chromosome 11, flanked proximally by *TYR* and distally by *EED* and *MYO7A* (See, Figure 3, Panel A). A conserved linkage group on mouse chromosome 7 was identified by comparison of the human genome map with the mouse genome mapping data (See, The Jackson Laboratory's web site for Mammalian Homology Query). Closer analysis of loci in the mouse conserved linkage group indicated that an uncloned mouse pigmentation mutant, *cht*, was contained within this interval (See, Figure 3, Panel A; and Potter and Rinchik, Mamm Genome 4:46-48 [1993]). The *cht* mutation arose as a spontaneous, isogenic mutation on an inbred C57B1/6J background and has been maintained on this background since 1984 (Macpike and Mobraaten, Mouse News Lett 700:86 [1984]). *Chl/cht* mice are identifiable at birth by lighter skin and eyes, and at weaning by a deep brown coat color when compared to the C57B1/6J parental strain (See, Figure 3, Panels B and C). Thus, *Rab38* was implicated as a candidate gene for the *cht* locus based on genomic map positions and the expression of *Rab38* in cell types affected in *cht/cht* mice, RPE and melanocyte derivatives.

#### **Mutation of *Rab38* Causes Melanocyte Defects in *cht/cht* Mice**

[0028] Genomic sequence flanking exon/intron boundaries for the three mouse *Rab38* exons was obtained from mouse trace archive genomic sequence (See, National Center for Biotechnology Information web site for Trace Archive Querying). DNA from C57B1/6J *chl+* animals was obtained from Jackson Laboratories Mouse DNA Resource, amplified using genomic *Rab38* primers and directly sequenced. In exon 1, a G146T nucleotide mutation was identified in the *cht* allele (See, Figure 4, Panel A). This sequence alteration was confirmed by restriction digest in multiple *cht/cht* DNA samples, as the resulting nucleotide substitution changes a *Bsa*JI site (CCNNGG) to a *SexA*1 restriction site (ACCWGGT) (See, Figure 4, Panel B). This sequence alteration was not detected in analysis of 8 additional inbred mouse strains (CAST/Ei, SPRET/Ei, 129/SVJ, FVB/NJ, AKR/J, A/J,

DBA/1J, BALB/cJ. The RAB38 protein demonstrates highly conserved amino acid identity between phyla: human/rat (96.2%), human/mouse (93.8%), rat/mouse (95.2%) (See, Figure 4, Panel B). The G19V *cht* mutation is located within the highly conserved phosphate/Mg<sup>2+</sup> (PM) domain and is predicted to directly contact GTP in the nucleotide binding pocket (See, Figure 5, Panels A and B).

### **Rab38<sup>cht</sup> Results in a Decrease in the Efficiency of Targeting Tyrp1 to End Stage Melanosomes**

[0029] Analysis of melanocytes cultured from newborn mice revealed that C57B1/6J *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* melanocytes contain, small, circular melanosomes with a brown hue similar to those observed in C57B1/6J *Tyrp1<sup>b</sup>/Tyrp1<sup>b</sup>* melanocytes (See, Figure 6, Panel C; and Hearing *et al.*, J Ultrastruct Res 43:88-106 [1973]), but distinct from the intensely black, oval melanosomes seen in C57B1/6J *+/+* melanocyte cultures (See, Figure 6, Panels A and B). Given that *Tyrp1* mutations cause the switch from black to brown pigment in *brown* mice, and given that Rab GTPases play a central role in protein trafficking (Schimmoller *et al.*, J Biol Chem 273:22161-22164 [1998]; and Chavrier and Goud, Curr Opin Cell Biol 11:466-475 [1999]), the targeting of TYRP1 to the melanosome was contemplated to be defective in *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* melanocytes. Consistent with this, endstage melanosomes in the *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* melanocytes stain much more weakly for TYRP1 than do end stage melanosomes in the control melanocytes (See, Figure 7, Panels A D). Additionally, GFP tagged RAB38 co-localizes with end stage melanosomes in wild type cells (See, Figure 7, Panels E and F). Thus, RAB38 is contemplated to regulate traffic of vesicular intermediates that move Tyrp1 from the trans-golgi network (TGN) to end stage melanosomes.

### **25 *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* Does Not Result in Platelet Storage Defects**

[0030] Subsets of mouse coat color mutants with mutations in genes involved in vesicular trafficking, such as *pale ear* and *beige*, also cause platelet aggregation defects modeling HPS and CHS respectively. To further analyze the pathology of *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* mice, bleeding times were measured as an assay of platelet function. No difference was observed between wildtype and *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* mice (2.53 vs. 2.41 minutes; p= 0.7). This observation is consistent with *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* being a genocopy for OCAIII (*Tyrp1<sup>b</sup>*) mouse model, but not for either HPS or CHS. Taken together, the data presented herein indicate that RAB38 is required for the efficient targeting of TYRP1 to pigmented melanosomes, and suggests that *Rab38<sup>cht</sup>/Rab38<sup>cht</sup>* is a genocopy of the *Tyrp1<sup>b</sup>/Tyrp1<sup>b</sup>* OCAIII mouse model.

***Rab38* is a Melanocyte Pigmentation Gene in Mammals**

[0031] Thus, by using cDNA microarray expression profiling, *Rab38* has been identified as an important gene involved in melanocyte pigmentation. Hierarchical clustering of expression patterns grouped *Rab38* with nine previously identified melanocyte genes known to function in a melanocyte specific fashion. These genes include *DCT*, *TRYPI*, and *PMELI7* which are essential for melanosome function; *MELAN-A/MART1* and *MLSN* which are important melanoma antigens (Chen *et al.*, Proc Natl Acad Sci USA 93:5915-5919 [1996]; and Duncan *et al.*, J Clin Oncol 19:568-576 [2001]); *AIM-1* which has recently been identified as the gene responsible for B in medaka (Fukamachi *et al.*, Nat Genet 28:381-385 [2001]), *underwhite* in mice (Newton *et al.*, Am J Hum Gent 69:981-988 [2001]), and *OCA4* in humans (Newton *et al.*, Am J Hum Gent 69:981-988 [2001]); *CHS1* which functions in melanosome/lysosome vesicle trafficking (Introne *et al.*, Mol Genet Metab 68:283-303 [1999]); and *PAX3*, a paired box transcription factor that regulates melanocyte gene expression (Watanabe *et al.*, Nat Genet 18:283-286 [1998]; Potterf *et al.*, Human Genet 107:1-6 [2000]; and Hornyak *et al.*, Mech Dev 101:47-59 [2001]), including expression of *TYRPI* (Galibert *et al.*, J Biol Chem 274:26894-26900 [1999]). Mutations in seven of these genes have been identified in human and/or marine disorders associated with variations in pigmentation (See, Figure 1).

[0032] *Rab38* was assessed as a candidate gene for the *cht* locus for three reasons. First, comparative genomic analysis predicted a co localization of the human *Rab38* gene to the region of the *cht* locus in the mouse genome. Second, the expression of *Rab38* was found to be restricted to those cell types affected in *cht/cht* mice (See, Figure 2, and Jager *et al.*, Cancer Res 60:3584-3591 [2000]). Finally, *Rab38* is a member of a family of proteins that are known to play a crucial role in vesicular trafficking (Nielsen *et al.*, Nat Cell Biol 1:376-382 [1999]; and Scott and Zhao, J Invest Dermatol 116:296-304 [2001]).

[0033] Sequence analysis of the *Rab38* coding region from *cht* mice revealed a G146T transversion in exon 1. This sequence alteration is likely to be the causative mutation, since this allele arose as a spontaneous mutation on a C57Bl/6J background. Moreover, the G146T alteration results in a Gly to Val substitution within the GTP binding pocket of RAB38. Crystal structure analysis of RAB3A, which is used as a model for other Rab proteins, predicts that this amino acid residue directly contacts the GTP within the nucleotide binding pocket (Dumas *et al.*, Structure Fold Des 7:413-423 [1999]). Furthermore, a mutation of the analogous amino acid residue in RAB5, a Rab that regulates the homotypic fusion of

endosomes, results in an increased rate of GDP dissociation *in vitro*, and the stimulation of endosome fusion *in vivo* (Li and Liang, Biochem J 355:681-689 [2001]). Additional support for the functional relevance of this mutation comes from studies of the Ras protein.

Substitutions in Ras at the analogous G13 residue, including the same G to V mutation as in *Rab38<sup>cht</sup>*, have been identified in acute myeloid leukemia (Bos *et al.*, Nature 315:726-730 [1985]; and Stirewalt *et al.*, Blood 97:3589-3595 [2001]). Based upon the analyses disclosed herein and on these observations, the Gly to Val mutation in RAB38 is contemplated to disrupt RAB38 function *in vivo*.

[0034] The coat color of *Rab38<sup>cht</sup>/Rab3<sup>cht</sup>* mice closely resembles that of the *brown* (*Tyrp1<sup>b</sup>/Tyrp1<sup>b</sup>*), OCAIII mouse model. The *brown* mouse model contains a defect in a melanin biosynthesis gene *Tyrp1*, resulting in a coat color change of the C57BL/6J mouse from black to brown. TYRP is a melanosomal membrane glycoprotein, which functions both as a DHICA oxidase enzyme and to provide structural stability to TYR in the melanogenic enzyme complex. TYRPI is believed to transit from the trans-golgi network (TGN) to stage II melanosomes via clatherin coated vesicles, possibly by first passing through an uncharacterized sorting compartment (Marks and Seabra, Nat Rev Mol Cell Biol 2:738-748 [2001]). Based upon the similar coat phenotype and predicted Rab protein function, RAB38 is contemplated to be specifically involved in trafficking of melanosomal proteins like TYRP1, to the melanosome. Consistent with this, GFP tagged RAB38 co localizes with melanosomes in pigmented melanocyte lines in culture and TYRP 1 is inefficiently targeted to pigmented end stage melanosomes in *Rab38<sup>cht</sup>/Rab3<sup>cht</sup>* melanocytes. The brown coat color observed in *Rab38<sup>cht</sup>/Rab3<sup>cht</sup>* mice is contemplated to be the result of a reduced amount of melanosomal TYRP1. Thus, RAB38 is implicated in the vesicle trafficking required for proper targeting of proteins, such as TYRP1, to melanosomes.

[0035] The formation of melanosomes and melanin pigment deposition within them requires a series of specific vesicular trafficking steps (King *et al.*, in Scriver *et al.* (eds.) The Metabolic Basis of Inherited Disease, 7th ed. (McGraw Hill, New York) pp.4353-4392 [1995]; and Marks and Seabra, Nat Rev Mol Cell Biol 2:738 748 [2001]). Comparison of the phenotype of *Rab38<sup>cht</sup>* mice to other mouse mutants where defects in the trafficking of proteins has been identified is contemplated to provide insight into the site of action of RAB38. Four genes involved in HPS (*HPS1*, *AP3* and *HPS3* and *HPS4*), when mutated result in the mouse models *pale ear* (*ep*), *mocha*, *cocoa*, and *light ear*, respectively. For each of these mouse models the color of melanin produced by the melanosome is lighter in color or of a brown hue. Interestingly, similar to what is seen in *Rab38<sup>cht</sup>/Rab3<sup>cht</sup>* derived

melanocytes, melanocytes from *Hps1<sup>ep</sup>/Hps1<sup>ep</sup>* mutants also exhibit a mislocalization of TYRP 1 into membraneous complexes rather than pre melanosomes (Sarangarajan *et al.*, J Invest Dermatol 117:641-646 [2001]), again yielding a brown mouse. In addition to melanosome pigment defects, HPS mice also exhibit enlargement of melanosomes and lysosomes, and reduced platelet cell aggregation (Swank *et al.*, Pigment Cell Res 13:59-67 [2000]; and Introne *et al.*, Mol Genet Metab 68:282-303 [1999]). This suggests the involvement of HPS genes in early vesicle sorting events that affect lysosomes, as well as melanosomes. However, it appears that *Rab38<sup>chl</sup>* is not in the same class of mutants as those of the HPS mouse models, since *Rab38<sup>chl</sup>/Rab38<sup>chl</sup>* mice do not exhibit enlarged melanosomes or lysosomes, or defects in platelet function. Thus, although both HPS1 and RAB38 appear to be involved in the proper sorting of TYRP1, this regulation appears to be occurring at different steps in the trafficking process. Since RAB38 appears to affect melanosome trafficking only, RAB38 is contemplated to be involved in vesicle trafficking downstream of the HPS genes.

[0036] *Rab38<sup>chl</sup>* mice appear to be a genocopy of the TYRP1<sup>b</sup>, OCA mouse model, due to the essential role of RAB38 in proper TYRP1 trafficking to late stage melanosomes, thus mimicking the cellular and clinical phenotype. OCA is a heterogeneous genetic disorder that has been associated with mutations in *TYR* (OCAI), *P* (OCAII) *TYRP1* (OCAIII) and *AIM1* (OCAIV). However, approximately 10% of patients clinically diagnosed with OCA do not have mutations in any of these genes. Given the heterogeneity of OCA and the predicted role of RAB38 in TYRP1 sorting, *Rab38* is contemplated to be a candidate gene for patients with OCA, particularly when a molecular defect in *TYR*, *P*, *TYRP1* or *AIM1* has not been found.

### High Throughput Screening for Modulators of RAB38 Activity

[0037] Conventionally, new chemical entities with useful properties are generated by identifying a chemical compound (called a "lead compound") with some desirable property or activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. However, the current trend is to shorten the time scale for all aspects of drug discovery. Because of the ability to test large numbers quickly and efficiently, high throughput screening (HTS) methods are replacing conventional lead compound identification methods.

[0038] In one preferred embodiment, high throughput screening methods involve providing a library containing a large number of potential therapeutic compounds (candidate compounds). Such "combinatorial chemical libraries" are then screened in one or more

assays, as described herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

5

a. ***Combinatorial chemical libraries***

[0039] Recently, attention has focused on the use of combinatorial chemical libraries to assist in the generation of new chemical compound leads. A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or  
10 biological synthesis by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks called amino acids in every possible way for a given compound length (*i.e.*, the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of  
15 chemical building blocks. For example, one commentator has observed that the systematic, combinatorial mixing of 100 interchangeable chemical building blocks results in the theoretical synthesis of 100 million tetrameric compounds or 10 billion pentameric compounds (Gallop *et al.* (1994) 37(9): 12331250).

[0040] Preparation and screening of combinatorial chemical libraries is well known to  
20 those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (*see, e.g.*, U.S. Patent 5,010,175, Furka (1991) *Int. J. Pept. Prot. Res.*, 37: 487-493, Houghton *et al.* (1991) *Nature*, 354: 84-88). Peptide synthesis is by no means the only approach envisioned and intended for use with the present invention. Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are  
25 not limited to: peptoids (PCT Publication No WO 91/19735, 26 Dec. 1991), encoded peptides (PCT Publication WO 93/20242, 14 Oct. 1993), random biooligomers (PCT Publication WO 92/00091, 9 Jan. 1992), benzodiazepines (U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, (1993) *Proc. Nat. Acad. Sci. USA* 90: 69096913), vinylogous polypeptides (Hagihara *et al.* (1992) *J. Amer. Chem. Soc.* 114: 6568), nonpeptidal peptidomimetics with a Beta D Glucose scaffolding (Hirschmann *et al.*,  
30 (1992) *J. Amer. Chem. Soc.* 114: 92179218), analogous organic syntheses of small compound libraries (Chen *et al.* (1994) *J. Amer. Chem. Soc.* 116: 2661), oligocarbamates (Cho, *et al.*, (1993) *Science* 261:1303), and/or peptidyl phosphonates (Campbell *et al.*, (1994) *J. Org. Chem.* 59: 658). *See, generally*, Gordon *et al.*, (1994) *J. Med. Chem.* 37:1385, nucleic acid

libraries, peptide nucleic acid libraries (*see, e.g.*, U.S. Patent 5,539,083) antibody libraries (*see, e.g.*, Vaughn *et al.* (1996) *Nature Biotechnology*, 14(3): 309-314), and PCT/US96/10287), carbohydrate libraries (*see, e.g.*, Liang *et al.* (1996) *Science*, 274: 1520-1522, and U.S. Patent 5,593,853), and small organic molecule libraries (*see, e.g.*, benzodiazepines, Baum (1993) C&EN, Jan 18, page 33, isoprenoids U.S. Patent 5,569,588, thiazolidinones and metathiazanones U.S. Patent 5,549,974, pyrrolidines U.S. Patents 5,525,735 and 5,519,134, morpholino compounds U.S. Patent 5,506,337, benzodiazepines 5,288,514, and the like).

[0041] Devices for the preparation of combinatorial libraries are commercially available (*see, e.g.*, 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony, Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA).

[0042] A number of well known robotic systems have also been developed for solution phase chemistries. These systems include automated workstations like the automated synthesis apparatus developed by Takeda Chemical Industries, LTD. (Osaka, Japan) and many robotic systems utilizing robotic arms (Zymate II, Zymark Corporation, Hopkinton, Mass.; Orca, HewlettPackard, Palo Alto, Calif.) which mimic the manual synthetic operations performed by a chemist. Any of the above devices are suitable for use with the present invention. The nature and implementation of modifications to these devices (if any) so that they can operate as discussed herein will be apparent to persons skilled in the relevant art. In addition, numerous combinatorial libraries are themselves commercially available (*see, e.g.*, ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, Columbia, MD, *etc.*).

b. ***High throughput assays of chemical libraries***

[0043] Any of the assays for compounds inhibiting the virulence described herein are amenable to high throughput screening. As described above, having identified the nucleic acid associated with virulence, likely drug candidates either inhibit expression of the gene product, or inhibit the activity of the expressed protein. Preferred assays thus detect inhibition of transcription (*i.e.*, inhibition of RAB38 mRNA production) by the test compound(s), inhibition of protein expression by the test compound(s), or binding to the gene (*e.g.*, gDNA, or cDNA) or gene product (*e.g.*, RAB38 mRNA or expressed protein) by the test compound(s). Alternatively, the assay can detect inhibition of the characteristic activity



of the gene product or inhibition of or binding to a receptor or other transduction molecule that interacts with the gene product.

[0044] High throughput assays for the presence, absence, or quantification of particular nucleic acids or protein products are well known to those of skill in the art. Similarly, binding assays are similarly well known. Thus, for example, U.S. Patent 5,559,410 discloses high throughput screening methods for proteins, U.S. Patent 5,585,639 discloses high throughput screening methods for nucleic acid binding (*i.e.*, in arrays), while U.S. Patents 5,576,220 and 5,541,061 disclose high throughput methods of screening for ligand/antibody binding.

[0045] In addition, high throughput screening systems are commercially available (*see, e.g.*, Zymark Corp., Hopkinton, MA; Air Technical Industries, Mentor, OH; Beckman Instruments, Inc. Fullerton, CA; Precision Systems, Inc., Natick, MA, *etc.*). These systems typically automate entire procedures including all sample and reagent pipetting, liquid dispensing, timed incubations, and final readings of the microplate in detector(s) appropriate for the assay. These configurable systems provide high throughput and rapid start up as well as a high degree of flexibility and customization. The manufacturers of such systems provide detailed protocols the various high throughput. Thus, for example, Zymark Corp. provides technical bulletins describing screening systems for detecting the modulation of gene transcription, ligand binding, and the like.

#### Methods of Inhibiting RAB38 Activity

[0046] Methods of the invention include methods of inhibiting RAB38 activity. Any method known in the art may be used, all that matters is that RAB38 activity be reduced or eliminated compared to a wild type state or a control. Inhibitors of RAB38 activity may act at any step of the expression process.

[0047] RAB38 activity may be inhibited by a number of methods including the use of ribozymes, antisense molecules, siRNA, or small chemical blockers of RAB38 activity.

#### Antisense and RNAi Polynucleotides

[0048] In certain embodiments, the activity of RAB38 protein is down-regulated, or entirely inhibited, by the use of an inhibitory or antisense polynucleotide, *e.g.*, a nucleic acid complementary to, and which can preferably hybridize specifically to, a coding mRNA nucleic acid sequence, *e.g.*, a RAB38 mRNA, or a subsequence thereof. Binding of the antisense polynucleotide to the mRNA reduces the translation and/or stability of the mRNA.

[0049] In the context of this invention, antisense polynucleotides can comprise naturally-occurring nucleotides, or synthetic species formed from naturally-occurring subunits or their close homologs. Antisense polynucleotides may also have altered sugar moieties or inter-sugar linkages. Exemplary among these are the phosphorothioate and other sulfur containing species. Analogs are comprehended by this invention so long as they function effectively to hybridize with the cancer protein mRNA. See, e.g., Isis Pharmaceuticals, Carlsbad, CA; Sequitor, Inc., Natick, MA.

[0050] Such antisense polynucleotides can readily be synthesized using recombinant means, or can be synthesized in vitro. Equipment for such synthesis is sold by several vendors, including Applied Biosystems. The preparation of other oligonucleotides such as phosphorothioates and alkylated derivatives is also well known.

[0051] Antisense molecules as used herein include antisense or sense oligonucleotides. Sense oligonucleotides can, e.g., be employed to block transcription by binding to the antisense strand. The antisense and sense oligonucleotide comprise a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target mRNA (sense) or DNA (antisense) sequences. A preferred antisense molecule is for a RAB38 molecules or for a ligand or activator thereof. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment generally at least about 14 nucleotides, preferably from about 14-30 nucleotides. The ability to derive an antisense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, e.g., Stein and Cohen (1988) Cancer Res. 48:2659-2668; and van der Krol, et al. (1988) BioTechniques 6:958-976.

[0052] RNA interference is a mechanism to suppress gene expression in a sequence specific manner. See, e.g., Brumelkamp, et al. (2002) Sciencexpress (21March2002); Sharp (1999) Genes Dev. 13:139-141; and Cathew (2001) Curr. Op. Cell Biol. 13:244-248. In mammalian cells, short, e.g., 21 nt, double stranded small interfering RNAs (siRNA) have been shown to be effective at inducing an RNAi response. See, e.g., Elbashir, et al. (2001) Nature 411:494-498. The mechanism may be used to downregulate expression levels of identified genes, e.g., treatment of or validation of relevance to disease.

[0053] In addition to antisense polynucleotides, ribozymes can be used to target and inhibit transcription of RAB38 nucleotide sequences. A ribozyme is an RNA molecule that catalytically cleaves other RNA molecules. Different kinds of ribozymes have been described, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNase P, and axhead ribozymes (see, e.g., Castanotto, et al. (1994) Adv. in Pharmacology 25: 289-317 for a general review of the properties of different ribozymes).

[0054] The general features of hairpin ribozymes are described, e.g., in Hampel, et al. (1990) Nucl. Acids Res. 18:299-304; European Patent Publication No. 0 360 257; U.S. Patent No. 5,254,678. Methods of preparation are described in, e.g., WO 94/26877; Ojwang, et al. (1993) Proc. Natl. Acad. Sci. USA 90:6340-6344; Yamada, et al. (1994) Human Gene Therapy 1:39-45; Leavitt, et al. (1995) Proc. Natl. Acad. Sci. USA 92:699-703; Leavitt, et al. (1994) Human Gene Therapy 5:1151-120; and Yamada, et al. (1994) Virology 205: 121-126.

[0055] Polynucleotide modulators of RAB38 may be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell. Alternatively, a polynucleotide modulator of RAB38 may be introduced into a cell containing the target nucleic acid sequence, e.g., by formation of an polynucleotide-lipid complex, as described in WO 90/10448. It is understood that the use of antisense molecules or knock out and knock in models may also be used in screening assays as, in addition to methods of treatment.

[0056] Intrabodies also provide effective means for blocking or modulating the activity of RAB38 proteins. The intracellularly expressed antibody constructs, usually single-chain Fv antibodies, are directed against a target inside a cell. The scFv gene can be transferred into cells, where scFv protein expression can modulate the properties of its target, e.g. RAB38, sometimes extinguishing protein function and causing a phenotypic knockout.

## **Topical Administration of RAB38 Inhibitors**

[0057] To be effective, a topical, RAB38 modulator or inhibitor should contain sufficient concentration of the active agent to produce an modulatory effect, where preferably the modulatory effect is an inhibitory effect, it should penetrate the tissue such as intact skin sufficiently to deliver a therapeutic dose, and it should exhibit rapid onset of action and have a prolonged effect. In achieving the foregoing, it is often desirable to have the RAB38 modulator present in a high concentration to effect a rapid onset and, additionally or alternatively, in excess of the amount that can be immediately absorbed through the dermis at the site of application, so as to prolong RAB38 modulation.

[0058] In the preferred topical formulations of the invention, the active RAB38 modulator is one which is generally suited to topical drug administration and includes any such materials known in the art. The topical carrier is selected so as to provide the composition in the desired form, e.g., as a liquid, lotion, cream, paste, gel, powder, or ointment, and may be  
5 comprised of a material of either naturally occurring or synthetic origin. It is essential, clearly, that the selected carrier not adversely affect the active agent or other components of the topical formulation. Examples of suitable topical carriers for use herein include water, alcohols and other nontoxic organic solvents, glycerin, mineral oil, silicone, petroleum jelly, lanolin, fatty acids, vegetable oils, parabens, waxes, and the like. The composition of the  
10 invention may also be administered in the form of a shampoo, in which case conventional components of such a formulation are included as well, e.g., surfactants, conditioners, viscosity modifying agents, humectants, and the like.

[0059] Particularly preferred formulations herein are colorless, odorless ointments, lotions, creams and gels.

[0060] Ointments are semisolid preparations which are typically based on petrolatum or other petroleum derivatives. The specific ointment base to be used, as will be appreciated by those skilled in the art, is one that will provide for optimum drug delivery, and, preferably, will provide for other desired characteristics as well, e.g., emolliency or the like. As with other carriers or vehicles, an ointment base should be inert, stable, nonirritating and  
20 nonsensitizing. As explained in Remington: The Science and Practice of Pharmacy, 19th Ed. (Easton, Pa.: Mack Publishing Co., 1995), at pages 1399-1404, ointment bases may be grouped in four classes: oleaginous bases; emulsifiable bases; emulsion bases; and water-soluble bases. Oleaginous ointment bases include, for example, vegetable oils, fats obtained from animals, and semisolid hydrocarbons obtained from petroleum. Emulsifiable ointment  
25 bases, also known as absorbent ointment bases, contain little or no water and include, for example, hydroxystearin sulfate, anhydrous lanolin and hydrophilic petrolatum. Emulsion ointment bases are either water-in-oil (W/O) emulsions or oil-in-water (O/W) emulsions, and include, for example, cetyl alcohol, glyceryl monostearate, lanolin and stearic acid. Preferred water-soluble ointment bases are prepared from polyethylene glycols of varying molecular  
30 weight; again, reference may be had to Remington: The Science and Practice of Pharmacy for further information.

[0061] Lotions are preparations to be applied to the skin surface without friction, and are typically liquid or semiliquid preparations in which solid particles, including the active agent, are present in a water or alcohol base. Lotions are usually suspensions of solids, and

preferably, for the present purpose, comprise a liquid oily emulsion of the oil-in-water type. Lotions are preferred formulations herein for treating large body areas, because of the ease of applying a more fluid composition. It is generally necessary that the insoluble matter in a lotion be finely divided. Lotions will typically contain suspending agents to produce better  
5 dispersions as well as compounds useful for localizing and holding the active agent in contact with the skin, e.g., methylcellulose, sodium carboxymethylcellulose, or the like. A particularly preferred lotion formulation for use in conjunction with the present invention contains propylene glycol mixed with a hydrophilic petrolatum such as that which may be obtained under the trademark Aquaphor.RTM. from Beiersdorf, Inc. (Norwalk, Conn.).

10 [0062] Creams containing the selected RAB38 modulator are, as known in the art, viscous liquid or semisolid emulsions, either oil-in-water or water-in-oil. Cream bases are water-washable, and contain an oil phase, an emulsifier and an aqueous phase. The oil phase, also sometimes called the "internal" phase, is generally comprised of petrolatum and a fatty alcohol such as cetyl or stearyl alcohol; the aqueous phase usually, although not necessarily,  
15 exceeds the oil phase in volume, and generally contains a humectant. The emulsifier in a cream formulation, as explained in Remington, supra, is generally a nonionic, anionic, cationic or amphoteric surfactant.

[0063] Gel formulations are may also be used. As will be appreciated by those working in the field of topical drug formulation, gels are semisolid, suspension-type systems. Single-  
20 phase gels contain organic macromolecules distributed substantially uniformly throughout the carrier liquid, which is typically aqueous, but also, preferably, contain an alcohol and, optionally, an oil.

[0064] Various additives, known to those skilled in the art, may be included in the topical formulations of the invention. For example, solvents may be used to solubilize certain drug  
25 substances. Other optional additives include skin permeation enhancers, opacifiers, anti-oxidants, gelling agents, thickening agents, stabilizers, and the like. Other agents may also be added, such as antimicrobial agents, antifungal agents, antibiotics and anti-inflammatory agents such as steroids.

[0065] In the preferred topical formulations of the invention, the active RAB38 modulator  
30 is present in an amount which is generally less than 10% by weight of the total composition, preferably less than about 1% by weight, and most preferably less than about 0.1% by weight. is present in an amount which is generally less than 10% by weight of the total composition, preferably less than about 1% by weight, and most preferably less than about 0.1% by weight.

## Definitions

[0066] To facilitate understanding of the invention, a number of terms are defined and discussed below.

5 [0067] The term "therapeutically effective amount" is intended to mean the amount of RAB38 modulator sufficient to produce an effect when applied topically. These amounts are known in the art or may be determined by methods known in the art, and typically range from about 1 to 20,000 mg per human adult and preferably about 10 to 10,000 mg and most preferably range from about 20 to 5,000 mg of the RAB38 modulatory agent per application, depending upon the RAB38 modulator chosen, and whether the tissue, such as the skin is the  
10 site of action.

[0068] The term "inhibitor of melanosme pigmentation" refers to any substance that blocks to any degree the formation of melanosomes and/or which blocks to any degree the deposition of pigment within the melanosomes. The inhibitor may act at any step of the processes leading to melanosome pigmentation including but not limited to, trafficking of  
15 melanosome vesicles, sorting of vesicles or proteins, or effects on protein activity.

[0069] The term "lightening of skin color" refers to a visible lessening of color, tone or shade of the skin, however slight. The change may be brought about by decreases in pigmentation of the melanosomes that arise as a result of reduced or inhibited RAB38 function.

20 [0070] "Inhibitors," "activators" or "modulators" of RAB38 polypeptide refer to inhibitory or activating molecules identified using *in vitro* and *in vivo* assays for RAB38 activity. Inhibitors are compounds that decrease, block, prevent, delay activation, inactivate, desensitize, or down regulate the activity. Activators are compounds that increase, open, activate, facilitate, enhance activation, sensitize or up regulate RAB38 activity. Such assays  
25 for inhibitors and activators include e.g., expressing a RAB38 polypeptide in a cell and then measuring GTPase activity. Alternatively, cells expressing endogenous RAB38 can be used in such assays. To examine the extent of inhibition, samples or assays comprising a RAB38 protein are treated with a potential activator or inhibitor and are compared to control samples without the inhibitor. Control samples (untreated with inhibitors) are assigned a relative  
30 RAB38 activity value of 100%. Inhibition of RAB38 activity is achieved when the RAB38 activity value relative to the control is about 90%, preferably 50%, more preferably 25-0%. Activation of RAB38 activity is achieved when the RAB38 activity value relative to the control is 110%, more preferably 150%, most preferably at least 200-500% higher or 1000% or higher.

[0071] The term "intrabody" or "intrabodies" refers to intracellularly expressed antibody constructs, usually single-chain Fv antibodies, directed against a target inside a cell. Nam, CH, et al. (2002) Methods Mol Biol. 193:301; der Maurr, AA, et al. (2002) J. Biol Chem Nov 22; 277(47):45075; Cohen, PA (2002) Methods Mol Biol 178:367. The scFv gene can be transferred into cells, where scFv protein expression can modulate the properties of its target, e.g. RAB38, sometimes extinguishing protein function and causing a phenotypic knockout. Indeed, the scFv intrabody can be expressed in the cytoplasm and directed to any cellular compartment where it can target intracellular proteins and elicit specific biological effects. Intrabodies thus provide effective means for blocking or modulating the activity of proteins.

[0072] The term "ribozyme" refers an RNA molecule that catalytically cleaves other RNA molecules. Different kinds of ribozymes have been described, including group I ribozymes, hammerhead ribozymes, hairpin ribozymes, RNase P, and axhead ribozymes (see, e.g., Castanotto, et al. (1994) Adv. in Pharmacology 25: 289-317 for a general review of the properties of different ribozymes).

[0073] The general features of hairpin ribozymes are described, e.g., in Hampel, et al. (1990) Nucl. Acids Res. 18:299-304; European Patent Publication No. 0 360 257; U.S. Patent No. 5,254,678. Methods of preparation are described in, e.g., WO 94/26877; Ojwang, et al. (1993) Proc. Natl. Acad. Sci. USA 90:6340-6344; Yamada, et al. (1994) Human Gene Therapy 1:39-45; Leavitt, et al.(1995) Proc. Natl. Acad. Sci. USA 92:699-703; Leavitt, et al. (1994) Human Gene Therapy 5:1151-120; and Yamada, et al. (1994) Virology 205: 121-126.

[0074] Any cellular gene product expressed as RNA, including proteins encoded by mRNA and structural RNAs themselves, can be targeted for specific cleavage and inactivation by ribozymes engineered to include appropriate regions of sequence and structure. New ribozymes have been engineered from the group I intron, either by directed evolution or by careful rational design, that cleave alternate substrates, including a DNA substrate.

[0075] The term "siRNA" refers to a small interfering RNA molecule which is part the RNA interference mechanism. RNA interference is a mechanism to suppress gene expression in a sequence specific manner. See, e.g., Brumelkamp, et al. (2002) Sciencexpress (21March2002); Sharp (1999) Genes Dev. 13:139-141; and Cathew (2001) Curr. Op. Cell Biol. 13:244-248. In mammalian cells, short, e.g., 21 nt, double stranded small interfering RNAs (siRNA) have been shown to be effective at inducing an RNAi response. See, e.g., Elbashir, et al. (2001) Nature 411:494-498. The mechanism may be used to downregulate expression levels of identified genes, e.g., to inhibit of RAB38 function for the purpose of decreasing skin pigmentation.

[0076] The terms "antisense molecule", "antisense oligonucleotide specific for RAB38", or "RAB38 antisense oligonucleotide" is an oligonucleotide having a sequence (i) capable of forming a stable triplex with a portion of a RAB38 nucleotide sequence, or (ii) capable of forming a stable duplex with a portion of an mRNA transcript of RAB38.

5 [0077] Antisense technology is emerging as an effective means of lowering the levels of a specific gene product. It is based on the findings that these "antisense" sequences hybridize a gene or associated target polynucleotide, to form a stable duplex or triplex, based upon Watson-Crick or Hoogsteen binding, respectively. The specifically bound antisense compound then either renders the respective targets more susceptible to enzymatic  
10 degradation, blocks translation or processing, or otherwise blocks or inhibits the function of a target polynucleotide. Where the target polynucleotide is RNA, the antisense molecule hybridizes to specific RNA transcripts disrupting normal RNA processing, stability, and translation, thereby preventing the expression of a targeted gene. Administration of antisense oligonucleotides or transfer of expression constructs capable of producing intracellular  
15 antisense sequences complementary to the mRNA of interest have been shown to block the translation of specific genes in vitro and in vivo. For example, Holt et al., Mol. Cell. Biol. 1988, 8, 963-973, focusing on c-myc, found the formation of an intra-cellular duplex with target mRNA and a selective decrease of c-myc protein in human promyelocytic leukemia HL-60 cells.

20 [0078] The term "oligonucleotide" as used herein refers to a polynucleotide formed from joined nucleotides. Moreover, the term "oligonucleotide" includes naturally occurring oligonucleotides or synthetic oligonucleotides formed from naturally occurring subunits or analogous subunits designed to confer special properties on the oligonucleotide so that it is more stable in biological systems or binds more tightly to target sequences. It also includes  
25 modifications of the oligonucleotides such as chemically linking them to other compounds that will enhance delivery to cells or to the nucleus and other compartments of cells. Further, oligonucleotides of the invention may contain modified internucleotide linkages to provide stability against nucleases. For example, the invention may include phosphorothioate oligodeoxyribonucleotides. Thus, the term "oligonucleotide" includes unmodified oligomers  
30 of ribonucleotides and/or deoxyribonucleotides, as well as oligomers wherein one or more purine or pyrimidine moieties, sugar moieties or internucleotide linkages is chemically modified.

[0079] Without limiting the generality of the foregoing, the term "oligonucleotide" as used herein includes linear oligomers of natural or modified monomers or linkages, including



deoxyribonucleosides, ribonucleosides, .alpha.-anomeric forms thereof, polyamide nucleic acids, and the like, capable of specifically binding to a target polynucleotide by way of a regular pattern of monomer-to-monomer interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Usually,

5 monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., 3-4, to several hundreds of monomeric units. Analogs of phosphodiester linkages include: phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like, as more fully described below. As used herein, "nucleoside" 10 includes the natural nucleosides, including 2'-deoxy and 2'-hydroxyl forms, e.g., as described in Kornberg and Baker, DNA Replication, 2nd Ed. (Freeman, San Francisco, 1992).

"Analog" in reference to nucleosides includes synthetic nucleosides having modified base moieties and/or modified sugar moieties, e.g., described generally by Scheit, Nucleotide 15 Analogs (John Wiley, New York, 1980). Such analogs include synthetic nucleosides designed to enhance binding properties, e.g., duplex or triplex stability, specificity, or the like.

It is preferred that the oligonucleotides of the invention be modified to increase stability and prevent intracellular and extracellular degradation. It is more preferred that the 20 oligonucleotides of the invention be modified to increase their affinity for target sequences, and their transport to the appropriate cells and cell compartments when they are delivered into a mammal in a pharmaceutically active form.

[0080] Target polynucleotides may be single-stranded or double-stranded RAB38 DNA or RAB38 RNA; however, single-stranded DNA or RNA targets are preferred. It is understood that the target to which the RAB38 antisense oligonucleotides of the invention are directed include allelic forms of RAB38. There is substantial guidance in the literature for selecting 25 particular sequences for antisense oligonucleotides given a knowledge of the sequence of the target polynucleotide, e.g., Peyman and Ulmann, Chemical Reviews, 90:543-584, 1990; Crooke, Ann. Rev. Pharmacol. Toxicol., 32:329-376 (1992); and Zamecnik and Stephenson, Proc. Natl. Acad. Sci., 75:280-284 (1974). Preferably, the sequences of RAB38 antisense compounds are selected such that the G-C content is at least 60%. Preferred proto-oncogene 30 mRNA targets include the 5'cap site, tRNA primer binding site, the initiation codon site, the mRNA donor splice site, and the mRNA acceptor splice site, e.g., Goodchild et al., U.S. Pat. No. 4,806,463.

[0081] Antisense oligonucleotides of the invention may comprise any polymeric compound capable of specifically binding to a target polynucleotide by way of a regular pattern of

monomer-to-nucleoside interactions, such as Watson-Crick type of base pairing, Hoogsteen or reverse Hoogsteen types of base pairing, or the like. Antisense compounds of the invention may also contain pendent groups or moieties, either as part of or separate from the basic repeat unit of the polymer, to enhance specificity, nuclease resistance, delivery, or other property related to efficacy, e.g., cholesterol moieties, duplex intercalators such as acridine, poly-L-lysine, "end-capping" with one or more nuclease-resistant linkage groups such as phosphorothioate, and the like.

[0082] For example, it is known that enhanced lipid solubility and/or resistance to nuclease digestion results by substituting an alkyl group or alkoxy group for a phosphate oxygen in the internucleotide phosphodiester linkage to form an alkylphosphonate oligonucleoside or alkylphosphotriester oligonucleotide. Non-ionic oligonucleotides such as these are characterized by increased resistance to nuclease hydrolysis and/or increased cellular uptake, while retaining the ability to form stable complexes with complementary nucleic acid sequences. The alkylphosphonates, in particular, are stable to nuclease cleavage and soluble in lipid. The preparation of alkylphosphonate oligonucleosides is disclosed in Tso et al., U.S. Pat. No. 4,469,863.

[0083] Preferably, nuclease resistance is conferred on the antisense compounds of the invention by providing nuclease-resistant internucleosidic linkages. Many such linkages are known in the art, e.g., phosphorothioate: Zon and Geiser, *Anti-Cancer Drug Design*, 6:539-568 (1991); Stec et al., U.S. Pat. No. 5,151,510; Hirschbein, U.S. Pat. No. 5,166,387; Bergot, U.S. Pat. No. 5,183,885; phosphorodithioates: Marshall et al., *Science*, 259:1564-1570 (1993); Caruthers and Nielsen, International application PCT/US89/02293; phosphoramidates, e.g., --OP (.dbd.O) (NR.sup.1 R.sup.2)--O-- with R.sup.1 and R.sup.2 hydrogen or C.sub.1 -C.sub.3 alkyl; Jager et al., *Biochemistry*, 27:7237-7246 (1988); Froehler et al., International application PCT/US90/03138; peptide nucleic acids: Nielsen et al., *Anti-Cancer Drug Design*, 8: 53-63 (1993), International application PCT/EP92/01220; methylphosphonates: Miller et al., U.S. Pat. No. 4,507,433, Ts'o et al., U.S. Pat. No. 4,469,863; Miller et al., U.S. Pat. No. 4,757,055; and P-chiral linkages of various types, especially phosphorothioates, Stec et al., European patent application 506,242 (1992) and Lesnikowski, *Bioorganic Chemistry*, 21:127-155 (1993). Additional nuclease linkages include phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, alkylphosphotriester such as methyl- and ethylphosphotriester, carbonate such as carboxymethyl ester, carbamate, morpholino carbamate, 3'-thioformacetal, silyl such as dialkyl (C.sub.1 -C.sub.6)- or diphenylsilyl, sulfamate ester, and the like. Such linkages

and methods for introducing them into oligonucleotides are described in many references, e.g., reviewed generally by Peyman and Ulmann, *Chemical Reviews* 90:543-584 (1990); Milligan et al., *J. Med. Chem.*, 36:1923-1937 (1993); Matteucci et al., International application PCT/US91/06855.

5 [0084] Resistance to nuclease digestion may also be achieved by modifying the internucleotide linkage at both the 5' and 3' termini with phosphoramidites according to the procedure of Dagle et al., *Nucl. Acids Res.* 18, 4751-4757 (1990).

[0085] Preferably, phosphorous analogs of the phosphodiester linkage are employed in the compounds of the invention, such as phosphorothioate, phosphorodithioate,  
10 phosphoramidate, or methylphosphonate. More preferably, phosphorothioate is employed as the nuclease resistant linkage.

[0086] Phosphorothioate oligonucleotides contain a sulfur-for-oxygen substitution in the internucleotide phosphodiester bond. Phosphorothioate oligonucleotides combine the properties of effective hybridization for duplex formation with substantial nuclease  
15 resistance, while retaining the water solubility of a charged phosphate analogue. The charge is believed to confer the property of cellular uptake via a receptor (Loke et al., *Proc. Natl. Acad. Sci.*, 86, 3474-3478 (1989)).

[0087] It is understood that in addition to the preferred linkage groups, compounds of the invention may comprise additional modifications, e.g., boronated bases, Spielvogel et al.,  
20 U.S. Pat. No. 5,130,302; cholesterol moieties, Shea et al., *Nucleic Acids Research*, 18:3777-3783 (1990) or Letsinger et al., *Proc. Natl. Acad. Sci.*, 86:6553-6556 (1989); and 5-propynyl modification of pyrimidines, Froehler et al., *Tetrahedron Lett.*, 33:5307-5310 (1992).

[0088] Oligonucleotides of the invention may be synthesized by any method known in the art. It is preferred in the present invention that the oligonucleotides be prepared using  
25 synthetic chemical methods, such as, for example, phosphoramidite chemistry by sulfurization with tetraethylthiuram disulfide in acetonitrile. See, for example, Vu and Hirschbein, *Tetrahedron Lett.* 1991, 32, 30005-30008. Oligonucleotides of the invention may also be synthesized using in vitro and in vivo transcription systems, such as transcription by T<sub>sup</sub>.7 polymerase or expression vectors. Oligonucleotides synthesized using in vitro and  
30 in vivo transcription systems may be modified via chemical methods known to those skilled in the art. Examples of such modifications include encapsulation in liposomes, or chemical linkage to steroids, antibodies, and cell receptor ligands.

[0089] In embodiments where triplex formation is desired, there are constraints on the selection of target sequences. Generally, third strand association via Hoogsteen type of

binding is most stable along homopyrimidine-homopurine tracks in a double stranded target. Usually, base triplets form in T-A\*T or C-G\*C motifs (where "-" indicates Watson-Crick pairing and "\*" indicates Hoogsteen type of binding); however, other motifs are also possible. For example, Hoogsteen base pairing permits parallel and antiparallel orientations between the third strand (the Hoogsteen strand) and the purine-rich strand of the duplex to which the third strand binds, depending on conditions and the composition of the strands. There is extensive guidance in the literature for selecting appropriate sequences, orientation, conditions, nucleoside type (e.g., whether ribose or deoxyribose nucleosides are employed), base modifications (e.g., methylated cytosine, and the like) in order to maximize, or otherwise regulate, triplex stability as desired in particular embodiments, e.g., Roberts et al., Proc. Natl. Acad. Sci., 88:9397-9401 (1991); Roberts et al., Science, 258:1463-1466 (1992); Distefano et al., Proc. Natl. Acad. Sci., 90:1179-1183 (1993); Mergny et al., Biochemistry, 30:9791-9798 (1992); Cheng et al., J. Am. Chem. Soc., 114:4465-4474 (1992); Beal and Dervan, Nucleic Acids Research, 20:2773-2776 (1992); Beal and Dervan, J. Am. Chem. Soc., 114:4976-4982; Giovannangeli et al., Proc. Natl. Acad. Sci., 89:8631-8635 (1992); Moser and Dervan, Science, 238:645-650 (1987); McShan et al., J. Biol. Chem., 267: 5712-5721 (1992); Yoon et al., Proc. Natl. Acad. Sci., 89:3840-3844 (1992); and Blume et al., Nucleic Acids Research, 20:1777-1784 (1992).

**[0090]** The length of the oligonucleotide moieties is sufficiently large to ensure that specific binding will take place only at the desired target polynucleotide and not at other fortuitous sites, as explained in many references, e.g., Rosenberg et al., International application PCT/US92/05305; or Szostak et al., Meth. Enzymol, 68:419-429 (1979). The upper range of the length is determined by several factors, including the inconvenience and expense of synthesizing and purifying large oligomers, the greater tolerance of longer oligonucleotides for mismatches than shorter oligonucleotides, whether modifications to enhance binding or specificity are present, whether duplex or triplex binding is desired, and the like.

**[0091]** It is preferred that the length of the oligonucleotide be between 5 and 200 nucleotides. It is more preferred that the oligonucleotide be between 10 and 50 nucleotides in length. It is most preferred that the oligonucleotide be between 15 and 25 nucleotides in length. In preferred embodiments, the oligonucleotide is specifically hybridizable with a translation initiation site. In one preferred embodiment of the present invention the oligonucleotide has the sequence 5' AACGTTGAGGGGCAT 3' (SEQ ID NO: 1). This

oligonucleotide is complementary to a segment of the RAB38 mRNA beginning with a translation initiation codon and extending downstream therefrom.

[0092] In general, the antisense oligonucleotides used in the practice of the present invention will have a sequence which is completely complementary to a selected portion of the target polynucleotide. Absolute complementarity is not however required, particularly in larger oligomers. Thus, reference herein to a "nucleotide sequence complementary to" a target polynucleotide does not necessarily mean a sequence having 100% complementarity with the target segment. In general, any oligonucleotide having sufficient complementarity to form a stable duplex with the target (e.g. the RAB38 mRNA) that is, an oligonucleotide which is "hybridizable", is suitable. Stable duplex formation depends on the sequence and length of the hybridizing oligonucleotide and the degree of complementarity with the target polynucleotide. Generally, the larger the hybridizing oligomer, the more mismatches may be tolerated. One skilled in the art may readily determine the degree of mismatching which may be tolerated between any given antisense oligomer and the target sequence, based upon the melting point, and therefore the thermal stability, of the resulting duplex.

[0093] Preferably, the thermal stability of hybrids formed by the antisense oligonucleotides of the invention are determined by way of melting, or strand dissociation, curves. The temperature of fifty percent strand dissociation is taken as the melting temperature,  $T_{sub.m}$ , which, in turn, provides a convenient measure of stability.  $T_{sub.m}$  measurements are typically carried out in a saline solution at neutral pH with target and antisense oligonucleotide concentrations at between about 1.0-2.0  $\mu$ M. Typical conditions are as follows: 150 mM NaCl and 10 mM  $MgCl_2$  in a 10 mM sodium phosphate buffer (pH 7.0) or in a 10 mM Tris-HCl buffer (pH 7.0). Data for melting curves are accumulated by heating a sample of the antisense oligonucleotide/target polynucleotide complex from room temperature to about 85-90.degree. C. As the temperature of the sample increases, absorbance of 260 nm light is monitored at 1.degree. C. intervals, e.g., using a Cary (Australia) model 1E or a Hewlett-Packard (Palo Alto, Calif.) model HP 8459 UV/VIS spectrophotometer and model HP 89100A temperature controller, or like instruments. Such techniques provide a convenient means for measuring and comparing the binding strengths of antisense oligonucleotides of different lengths and compositions.

[0094] According to one embodiment, the oligonucleotides of this invention are designed to be hybridizable with messenger RNA derived from the RAB38 gene. Such hybridization, when accomplished, interferes with the normal roles of the messenger RNA to cause a modulation of its function in the cell. The functions of messenger RNA to be interfered with

include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to modulate

5 expression of the RAB38 gene. By modulating the expression of RAB38, melanocyte pigmentation is modulated, or inhibited.

[0095] The terms "nucleic acid," "nucleic acid sequence," and "nucleotide sequence," as used herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which can be single or double stranded,  
10 and represent the sense or antisense strand.

[0096] As used herein, the terms "restriction endonuclease" and "restriction enzyme" refer to bacterial enzymes, each of which cut double stranded DNA at or near a specific nucleotide sequence, referred to as a "restriction site."

[0097] As used herein, the terms "complementary" or "complementarity" are used in  
15 reference to antiparallel polynucleotides (i.e., a sequence of nucleotides) related by the base pairing rules. For example, the sequence 5' AGTTC-3' is complementary to the sequence 3'-TCAAG 5'.

[0098] "Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) or other  
20 technologies well known in the art (e.g., Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY [1995]). As used herein, the term "polymerase chain reaction" ("PCR") refers to the Mullis method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification (See e.g., U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188,  
25 hereby incorporated by reference). This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the  
30 primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension (DNA synthesis) are typically reiterated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there usually are numerous "cycles") to obtain a high

concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be VCR amplified."

[0099] As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete.

[0100] The terms "peptide," "polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent peptide linkages. In general, a peptide consists of a few amino acids, typically from 2-25 amino acids, and is shorter than a protein.

"Polypeptides" encompass both peptides and proteins.

[0101] As used herein the term "portion" when in reference to a gene refers to fragments of that gene. In some embodiments, the fragments range in size from ten nucleic acids to the entire nucleic acid sequence minus one nucleic acid.

[0102] As used herein, the term "purify" or "purifying" refers to the removal of at least one contaminant from a sample. As used herein, the term "substantially purified" refers to molecules, either nucleic acids or amino acid sequences, that are removed from their natural environment, "isolated" or "separated," and are largely free from other components.

[0103] The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence comprised of parts, that when appropriately combined in either a native or recombinant manner, provide some product or function. In addition to the coding region of the nucleic acid, the term "gene" also encompasses the transcribed nucleotide sequences of the full-length mRNA adjacent to the 5' and 3' ends of the coding region. These noncoding regions are referred to as 5' and 3' untranslated sequences (5' UT and 3' UT). Both the 5' and 3' UT may serve regulatory roles, including translation initiation, post transcriptional cleavage and polyadenylation. In preferred embodiments, a mammalian "Rab38 gene" is provided.

[0104] In some embodiments, the "genomic" form of a gene contains the sequences of the transcribed mRNA, as well as other non coding sequences. "Introns" or "intervening sequences" are segments of a gene which are contained in the primary transcript (*i.e.*, heteronuclear RNA, or hnRNA), but are spliced out to yield the processed mRNA form.

Conversely, "exons" are the segments of a gene corresponding to the processed mRNA sequence.

[0105] The terms "in operable combination," and "operably linked" when used in reference to nucleic acid herein are used to refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. In preferred embodiments of the present invention, a mammalian *Rab38* gene in "operable combination" with a promoter and/or an enhancer is provided.

[0106] As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segments) from one cell to another. In some embodiments, a vector "backbone" comprises those parts of the vector which mediate its maintenance and enable its intended use (e.g., sequences necessary for replication, genes imparting drug or antibiotic resistance, a multiple cloning site, and possibly operably linked promoter/enhancer elements which enable the expression of a cloned nucleic acid). Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

[0107] As used herein, the term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene.

[0108] In contrast, the terms "mutant" and "mutation" refer to a gene or gene product which displays modifications in sequence and or functional properties (i.e., altered characteristics) when compared to the wild type gene or gene product. It is noted that naturally-occurring mutants can be isolated and these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product. In some embodiments, the present invention provides a mutant *Rab38* gene or RAB38 protein. In preferred embodiments, the *chocolate Rab38*/RAB38 mutant is provided.

[0109] The term "candidate agent" refers to any molecule of any composition, including proteins, peptides, nucleic acids, lipids, carbohydrates, organic molecules, inorganic molecules, and/or combinations of molecules which are suspected to be capable of producing a physiological or biological response.

[0110] As used herein, the term "modulate" refers to a change in the activity of RAB38. For example, modulation may cause an increase or a decrease in enzymatic activity, binding characteristics, or any other biological, or functional properties of RAB38.



[0111] The term "melanocyte" as used herein, refers to special cells in the skin and the eye that synthesize melanin pigments. Clusters of melanocytes often appear on the skin as moles. The term "melanosome" refers to the melanin producing organelle of melanocytes.

[0112] As used herein, the terms "GTPase activity" and "guanosine triphosphatase activity" refer to the enzyme activity that hydrolyses GTP to produce GDP and orthophosphate. GTPase activity is regulated by GTPase activating proteins (activation) and by guanine nucleotide releasing proteins (inhibition). In the context of the invention, GTPase activity refers to "RAB38 activity" or the GTPase activity of RAB38. RAB38 is inactive when bound to GDP, and active when bound to GTP. Thus, the term "GTP binding" refers to the binding of GTP by a GTPase (*e.g.*, RAB38), while the term "GDP release" refers to the release of GDP by a GTPase (*e.g.*, RAB38). GTPases of the RAB family have been implicated in the process of vesicle trafficking. In the context of the invention, the term "RAB activity" encompasses "RAB38 trafficking" or the transport of RAB38 to melanosomes of melanocytes, and "TYRP1 trafficking" or the transport of TYRP1 to melanosomes.

[0113] As used herein the term "intradermal-penetration agent" means an agent capable of transporting a pharmacologically active compound through the stratum corneum and into the epidermis or dermis, while keeping the pharmacological effects restricted to the intracutaneous regions of drug penetration, preferably, with little or no systemic absorption.

[0114] A "penetration enhancing amount" of an intradermal-penetration agent is an amount which enhances the RAB38 inhibitor or modulator penetration rate through the stratum corneum, relative to the penetration rate without the intradermal-penetration agent.

[0115] The term "pharmaceutically acceptable topical formulation" as used herein means any formulation which is pharmaceutically acceptable for topical administration of a RAB38 modulator or inhibitor by application of the formulation to the epidermis. According to the invention, a "topical formulation" will comprise at least one RAB38 modulator or inhibitor and may also include an intradermal-penetration agent. The choice of topical formulation will depend on several factors, including the skin condition to be treated, and other excipients present, their stability in the formulation, available manufacturing equipment, and costs constraints.

## EXPERIMENTAL

[0116] The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

[0117] In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); BSA (bovine serum albumin); DMEM (Dulbecco's modified eagle's medium); FBS (fetal bovine serum); H<sub>2</sub>O (water); aa (amino acid); bp (base pair); kb (kilobase pair); EST (expressed sequence tag); kD (kilodaltons); gm (grams); µg (micrograms); mg  
5 (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); CO<sub>2</sub> (carbon dioxide); Cy3 (indocarbocyanine); Cy5 (indodicarbocyanine); (DEPC (diethyl pyrocarbonate); dNTPs (deoxynucleotides); MgCl<sub>2</sub> (magnesium chloride); PBS (phosphate  
10 buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); SDS (sodium dodecyl sulfate); SSC (saline sodium citrate buffer); TBS (Tris buffered saline); PCR (polymerase chain reaction); RT (reverse transcription); w/v (weight to volume); v/v (volume to volume); Applied Biosystems (Applied Biosystems, Foster City, CA); Axon (Axon Instruments Inc., Foster City, CA); GeneCodes (GeneCodes, Ann Arbor, MI); Invitrogen  
15 (Invitrogen Corp., Carlsbad, CA); Jackson (Jackson Laboratory, Bar Harbor, ME); NEB (New England Biolabs, Inc., Beverly, MA); NIH (National Institutes of Health, Bethesda, MD); Qiagen (Qiagen, Valencia, CA); Roche (Roche, MannHeim, Germany); and Signet (Signet Laboratories, Dedham, MA).

## EXAMPLE 1

### Cell Culture

[0118] All cells for microarray analysis were grown to 90% confluence at 37°C in 5% CO<sub>2</sub>. The cell lines used in these experiments were obtained from Jeffry Trent (NIH). Melanoma cell lines were grown in RPMI media containing 10% FBS, 2 mM L glutamine and 100  
25 units/ml each of penicillin and streptomycin. Media for MnSOD6 cl1 also contained 500 µg/ml geneticin. 293T, U138 and HeLa cells were grown in DMEM media containing 10% FBS, 2mM L glutamine and 100 units/ml each of penicillin and streptomycin. Primary murine melanocytes were cultured as previously described (Wu *et al.*, J Cell Sci 114:1091-1100 [2001]. Melan-a cells were cultured in RPMI 1640 media containing 10% FBS, 2 mM  
30 L-glutamine, 10 mM sodium pyruvate, 100 units/ml each of penicillin and streptomycin, 200 nm 12-O-tetradecanoylphorbol 13-acetate, 0.01 mM sodium bicarbonate and 0.1 mM 2-mercaptoethanol in 5% CO<sub>2</sub>.

## EXAMPLE 2

### RNA Preparation

[0119] Cells for microarray analysis were obtained in pools of four 500 cm<sup>2</sup> dishes, that were harvested by scraping, washed in PBS, and pelleted. Pellets were lysed in 10 ml Trizol reagent (Invitrogen). Two ml of chloroform were added, the sample was shaken, and then centrifuged to separate phases. The aqueous layer was removed and an equal volume of 75% ethanol was added dropwise while vortexing. Sample was applied to a RNeasy maxi column (Qiagen) and the manufacturer's purification protocol was followed. Samples were eluted in water, precipitated with 3 M sodium acetate and stored at - 80°C. RNA pellets were resuspended in DEPC water to 1 µg/p,l concentration, and applied to a Microcon 100 column. RNA samples were centrifuged and concentrated to 7 10 µg/ul.

## EXAMPLE 3

### Labeling and Hybridization

[0120] RNA was reverse transcribed to fluorescent labeled cDNA and co hybridized on slides in experimental/reference pairs. Expressed sequence tag (EST) clone inserts were prepared and applied to slides as described (DeRisi *et al.*, Nat Genet 14:457-460 [ 1996]). Reversed transcribed (RT) fluorochrome labeled cDNA was generated as known in the art (See, The National Human Genome Research Initiative's Microarray Project web site). For reactions, 60 µg of total RNA (Cy3) or 120 µg of total RNA (Cy5) were used. Hybridizations were carried out in a final volume of 40 µl at 65°C in a humidified chamber for 16 hr. Slides were washed at room temperature in 0.5X SSC/0.1% SDS for 3 min followed by a second wash in 0.6X SSC for 3 min. Slides were immediately spun dry by centrifugation.

## EXAMPLE 4

### Image Acquisition and Analysis

[0121] Fluorescence signal intensities for Cy3 (532 λ) and Cy5 (635 λ) fluorochromes were obtained using a Genepix 4000a scanner (Axon) at 10 µM resolution. A set of 88 housekeeping control genes was used to normalize for labeling efficiency (Loftus *et al.*, Proc Natl Acad Sci USA 96:9277-9280 [1999]). Expression profile analysis was performed with a clustering algorithm using average linkage method and Pearson's correlation similarity measurement (See, The National Human Genome Research Initiative's Genome Clustering web site).

**EXAMPLE 5****Organization of the Mouse *Rab38* Gene**

[0122] To facilitate mutation screening of the mouse *Rab38* gene, database searches were employed to identify genomic DNA adjacent to *Rab38* coding sequences. *Rab38* mRNA sequence (SEQ ID NO:9 and GenBank Accession No. AY062237), was BLASTed against mouse genomic sequencing trace archives (*See*, The National Center for Biotechnology Information's web site for Trace Archive Querying). Significant similarities were determined by a returned BLAST score of greater than 200. Relevant data were downloaded and aligned to the mRNA sequence using Sequencher version 3.1.1 (GeneCodes). Genomic organization was confirmed by using the Spidey program (*See*, The National Center for Biotechnology Information's web site for Spidey). Gene organization was also experimentally confirmed through PCR and DNA sequencing of genomic fragments. This analysis revealed that *Rab38* is composed of three exons: exon 1, nucleotides 1-292; exon 2, nucleotides 292-572; and exon 3, nucleotides 573-1439 (nucleotide positions refer to GenBank Accession No. AY062237). The confirmed exon and surrounding intron sequences have been deposited into GenBank as: exon 1, AF448441 (SEQ ID NO:10); exon 2, AF448442 (SEQ ID NO: 11); and exon 3, AF448443 (SEQ ID NO:12).

**EXAMPLE 6*****In Situ* Hybridization**

[0123] Timed matings were used to obtain staged FVBINJ (Jackson) mouse embryos, and EO.5 was designated as noon on the day of vaginal plug formation. Embryos were fixed overnight in 4% paraformaldehyde in PBS. Digoxigenin conjugated probes were synthesized by reverse transcription (RT) of linearized plasmids and/or PCR products with RT binding site linkers (Roche). The following DNA sources were used for probe synthesis: Tyrosinase, cDNA clone 4633402007; *Tyrp1*, RT-PCR from B16 cell line total RNA (TYRP15'T3F 5'-GCGCGAATTA ACCCTCACTA AAGGGTCTGA GCACCCCTGT CTTCT 3', SEQ ID NO:13; TYRP15'T7R 5' -GCGCGTAATACGACTCACTA TAGGGCCCAG TTGCAAAATT CCAGT-3', SEQ ID NO:14); *Dct*, cDNA; *Aim1/Matp*, RIKEN cDNA clone G370045L22; *Mlsnl* RT-PCR from B16 cell line total RNA (MLSN R T7 5' GCGGGTAATA CGACTCACTA TAGGGGCCAC AAACATGTCC TACTTAC-3', SEQ ID NO:15; MLSN FT3 5' GCGCGAATTA ACCCTCACTA AAGGGAAGCT TCCGGACTCT CTAC 3', SEQ ID NO:16); *Rab38*, Riken cDNA clone 23 10011 F14. *In situ* hybridizations were performed using published protocols (Wilkinson and Nieto, *Methods Enzymol* 225:361-373 [1993]),

with the following modifications. After probe hybridization, Ribonuclease A digestion was omitted, TBS was used in place of PBS, and the substrate BM-purple (Roche) was used in place of 5-bromo-4 chloro-3-indolyl phosphate/nitro blue tetrazolium.

## EXAMPLE 7

### Mutation Detection

[0124] The mouse *Rab38* gene homologue was screened for mutations by PCR amplification and DNA sequencing of exon containing genomic segments. The following primer pairs were designed to amplify the three protein coding exons, as well as a small amount of flanking intron DNA: *Rab38* Ex1F 5'-TAGGAAGGAGGATTAAACCC G 3' (SEQ ID NO:17) and *Rab38* Ex 1R 5' GAACTCCTCATGGCTCACTC C 3' (SEQ ID NO:18) yielding a 428 bp product; *Rab38* Ex2F 5'-GGATATGAAGCTCCAGTGTA GTGTAC 3' (SEQ ID NO:19) and *Rab38* Ex2R 5'CACTGGACAG AAACATTATT GTCAC 3' (SEQ ID NO:20) yielding a 464 bp product; and *Rab38* Ex3F 5'-AAGTTATCAG CCAGTGAGAT ACTGTG 3' (SEQ ID NO:21) and *Rab38* Ex3R 5'-CACATGTGGT ATATCTATCC TGACG 3' (SEQ ID NO:22) yielding a 526 bp product. PCR reactions contained 1.5  $\mu$ M of each primer, 0.2  $\mu$ M dNTPs, 1.5 pM  $MgCl_2$ , 1 unit AmpliTaq DNA Polymerase (Applied Biosystems), 1X of the manufacturer's IOX buffer, and 40 ng of mouse genomic DNA. Thermal cycling consisted of an initial denaturation for 2 min at 93°C, followed by 40 cycles of 93°C for 10 sec, 55°C for 5 sec, and 72°C for 30 sec. A final extension at 72°C was performed for 7 min. Following separation on a 1% agarose gel, PCR products were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). DNA sequencing was performed with BigDye terminator chemistry, and a model 3100 DNA sequencing instrument (Applied Biosystems). The cycle-sequencing routine was 30 cycles of 92°C for 20 sec, 55°C for 10 sec, and 60°C for 4 min with a 20  $\mu$ L reaction containing 8  $\mu$ L of BigDye cocktail, 0.5  $\mu$ L of a 25  $\mu$ M primer solution, 6.5  $\mu$ L of water, and 5  $\mu$ L of PCR product (at  $\sim$ 50 ng/ $\mu$ L). Data was extracted and analyzed using Sequencing Analysis version 3.3 (Applied Biosystems) and aligned with Sequencher software version 3.1.1 (GeneCodes). Alignments included sequence data derived from wildtype C57B1/6J and heterozygous *chl*<sup>+</sup> mice, as well as mouse *Rab38* mRNA sequence (GenBank Accession No. AY062237).

## EXAMPLE 8

### Mutation Confirmation and Genotyping

[0125] Primers were designed to amplify a 213 bp fragment surrounding the G146T sequence change (cht Ex1F 5' -GGCCTCCAGG ATGCAGACAC C 3', SEQ ID NO:23; cht Ex1R 5' CCAGCAATGT CCCAGAGCTG C 3', SEQ ID NO:24). PCR amplification was performed as described in Example 7. *SexAI* and *BsaJI* restriction digests were performed using 20  $\mu$ L restriction enzyme digests containing 10  $\mu$ L of PCR product, 2.5 units enzyme (NEB) along with 1X of the supplied BSA and digest buffer. Reactions were incubated overnight at the manufacturer's suggested temperature and electrophoresed on a 2% agarose gel to visualize band patterns.

## EXAMPLE 9

### Cell Transfection

[0126] GFP-RAB38 constructs were generated by PCR amplifying mouse *Rab38* with *att* site linker primers (AttB1-RRab 5'-GGGGACAAGT TTGTACAAAA AAGCAGGCTC CATGCAGACA CCTCACAAG -', SEQ ID NO:25 and AttB2-RRab-STP 5' - GGGGACCACT TTGTACAAGA AAGCTGGGTT CTAGGATTTG GCACAGCCAG A 3', SEQ ID NO:26) and "Gateway" cloning into pDest 53 (Invitrogen) as per manufacturer's instructions. GFP-RAB38 was transfected into melan-a cells using LipofectAMINE 2000 (Invitrogen) with a DNA / LipofectAMINE 2000 ratio of 1.6  $\mu$ g/ $\mu$ L in a 4 cm<sup>2</sup> surface area as per manufacturer's instructions. After 72 hours, cells were fixed and stained with a 1:200 dilution of the TYRP1-reactive antibody MEL5 (Signet) as previously described (Wu *et al.*, J Cell Sci 110:847-859 [1997]).

## EXAMPLE 10

### Bleeding Times

[0127] Bleeding times were assayed in four C57Bl/6J *Rab38<sup>chl</sup>/Rab38<sup>chl</sup>* and four C57Bl/6J animals, as described in the art (Sviderskaya *et al.*, Genetics 148:381-390 [1998]). Assayed mice were 6-12 weeks of age. A 2 mm portion of the tail was removed and the cut tail immediately immersed in saline at 37°C. Each mouse was maintained in a horizontal position in a restrainer with the tip of the tail held 4-5 cm below the body. Bleeding time was that required for the small stream of blood to stop abruptly.

[0128] All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and

system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and/or related fields are intended to be within the scope of the present invention. pDest 53 (Invitrogen) as per manufacturer's instructions. GFP RAB38 was transfected into melan a cells using LipofectAMINE 2000 (Invitrogen) with a DNA / LipofectAMINE 2000 ratio of 1.6 g/4 µl in a 4 cm' surface area as per manufacturer's instructions. After 72 hours, cells were fixed and stained with a 1:200 dilution of the TYRP1 reactive antibody MELS (Signet) as previously described (Wu *et al.*, J Cell Sci 110:847-859 [1997]).

WHAT IS CLAIMED IS:

- 1                   1.       An isolated nucleic acid comprising a sequence selected from the  
2   group consisting of SEQ ID NO:9, SEQ ID NO: 10, SEQ ID NO: 11, and SEQ ID NO:12.
- 1                   2.       The isolated nucleic acid of Claim 1, wherein said nucleic acid is  
2   deoxyribonucleic acid.
- 1                   3.       An isolated nucleic acid, wherein said nucleic acid is the complement  
2   of the nucleic acid of Claim 1.
- 1                   4.       A vector comprising the nucleic acid of Claim 1.
- 1                   5.       A host cell comprising the vector of Claim 4.
- 1                   6.       An isolated protein encoded by the nucleic acid sequence set forth in  
2   SEQ ID NO:9.
- 1                   7.       A method for detecting mutations in *Rab38* comprising the steps of:  
2                   a)       amplifying at least a portion of *Rab38* from genomic DNA to  
3   yield a *Rab38* amplification product;  
4                   b)       purifying said *Rab38* amplification product; and  
5                   c)       sequencing said *Rab38* amplification product.
- 1                   8.       The method of Claim 7, wherein said amplifying is accomplished  
2   using a polymerase chain reaction.
- 1                   9.       The method of Claim 7, wherein said at least a portion of *Rab38* is  
2   selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the  
3   *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence.
- 1                   10.      The method of Claim 9, wherein said at least one *Rab38* exon is  
2   selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3.
- 1                   11.      The method of Claim 7, wherein said genomic DNA is mammalian  
2   genomic DNA.
- 1                   12.      The method of Claim 7, wherein said purifying is accomplished using  
2   size selection.



1                   13.    A method for detecting mutations in *Rab38* comprising the steps of:  
2                   a)     amplifying at least a portion of *Rab38* from genomic DNA to  
3 yield a *Rab38* amplification product;  
4                   b)     digesting said *Rab38* amplification product to yield a digested  
5 *Rab38* amplification product; and  
6                   c)     electrophoresing said digested *Rab38* amplification product.

1                   14.    The method of Claim 13, wherein said amplifying is accomplished  
2 using a polymerase chain reaction.

1                   15.    The method of Claim 13, wherein said at least a portion of *Rab38* is  
2 selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the  
3 *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence.

1                   16.    The method of Claim 15, wherein said at least one *Rab38* exon is  
2 selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3.

1                   17.    The method of Claim 13, wherein said genomic DNA is mammalian  
2 genomic DNA.

1                   18.    A method for screening for biologically active agents to modulate  
2 RAB38 activity, comprising the steps of:

3                   a)     providing:  
4                   i)     melanocytes comprising RAB38 activity, and  
5                   ii)    a candidate agent; and  
6                   b)     exposing said melanocytes to said candidate agent to yield  
7 treated melanocytes; and  
8                   c)     measuring the modulation of said RAB38 activity of said  
9 treated melanocytes by said candidate agent.

1                   19.    The method of Claim 18, wherein said RAB38 activity comprises  
2 GTPase activity.

1                   20.    The method of Claim 18, wherein said RAB38 activity comprises GTP  
2 binding activity.

1                   21.     The method of Claim 18, wherein said RAB38 activity comprises GDP  
2 release.

1                   22.     The method of Claim 18, wherein said RAB38 activity comprises  
2 TYRP1 trafficking to melanosomes.

1                   23.     The method of Claim 18, wherein said RAB38 activity comprises  
2 RAB38 trafficking to melanosomes.

1                   24.     A kit for screening for biologically active agents that modulate RAB38  
2 activity, comprising: a) plurality of melanocytes comprising RAB38 activity, wherein said  
3 melanocytes are provided within a container, and b) instructions for determination of RAB38  
4 activity in said melanocytes.

1                   25.     The kit of Claim 24, further comprising means to analyze RAIB38  
2 activity.

1                   26.     The kit of Claim 25, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GTPase activity.

1                   27.     The kit of Claim 25, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GTP binding activity.

1                   28.     The kit of Claim 25, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GDP release.

1                   29.     The kit of Claim 25, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess TYRP 1 trafficking to melanosomes.

1                   30.     The kit of Claim 25, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess RAB38 trafficking to melanosomes.

1                   31.     A kit for detection of mutations in RAB38 comprising at least two  
2 primer sequences suitable for amplification of at least a portion of RAB38, and instructions  
3 for utilizing said kit.

1                   32.     The kit of Claim 31, wherein said primer sequences are selected from  
2 the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20,  
3 SEQ ID NO:21, and SEQ ID NO:22.

1                   33.     The kit of Claim 31, wherein said kit is suitable for use in the  
2 polymerase chain reaction.

1                   34.     The kit of Claim 31, further comprising reagents for digesting nucleic  
2 acid.

1                   35.     A kit for diagnosing defects in melanosome function, comprising  
2 melanocytes comprising *RAB38* and instructions for assessing defects in melanosome  
3 function.

1                   36.     The kit of Claim 35, further comprising means to analyze RAB38  
2 activity.

1                   37.     The kit of Claim 36, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GTPase activity.

1                   38.     The kit of Claim 36, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GTP binding activity.

1                   39.     The kit of Claim 36, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess GDP release.

1                   40.     The kit of Claim 36, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess TYRP1 trafficking to melanosomes.

1                   41.     The kit of Claim 36, wherein said means to analyze RAB38 activity  
2 comprises an assay to assess RAB38 trafficking to melanosomes.

1                   42.     A composition for modulating pigmentation of melanocytes,  
2 comprising a modulator of RAB38 activity.

1                   43.     The composition of claim 42, wherein the modulator of RAB38  
2 activity is an enhancer of RAB38 activity.

1                   44.     The composition of claim 42, wherein the modulator of RAB38  
2 activity is an inhibitor of RAB38 activity.

1                   45.     The composition of claim 44, wherein the inhibitor of RAB38 activity  
2 is selected from the group consisting of siRNA and intrabodies.

1                   46.     A method of modulating the pigmentation of a melanosome and  
2 changing skin color, the method comprising: contacting a skin surface with a modulator of  
3 RAB38 activity, thereby regulating the activity of RAB38.

1                   47.     The method of claim 46, wherein the modulator is an inhibitor of  
2 RAB38 activity that down-regulates RAB38 activity and lightens skin color.

1                   48.     The method of claim 46, wherein the modulator is an enhancer of  
2 RAB38 activity that up-regulates RAB38 activity and darkens skin color.

1/7

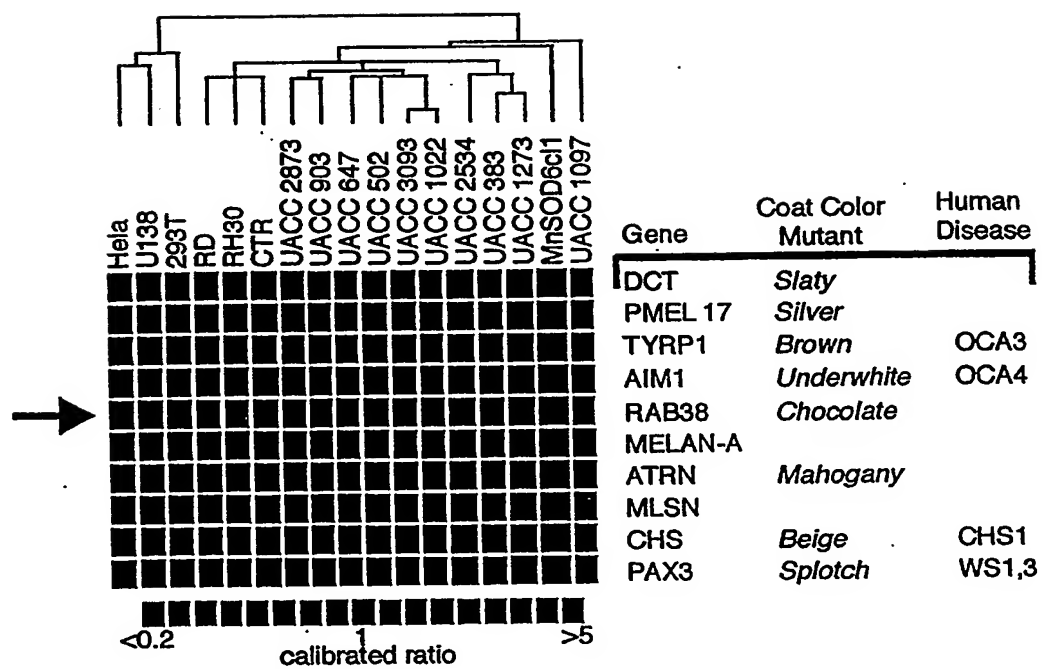


Fig. 1

2/7

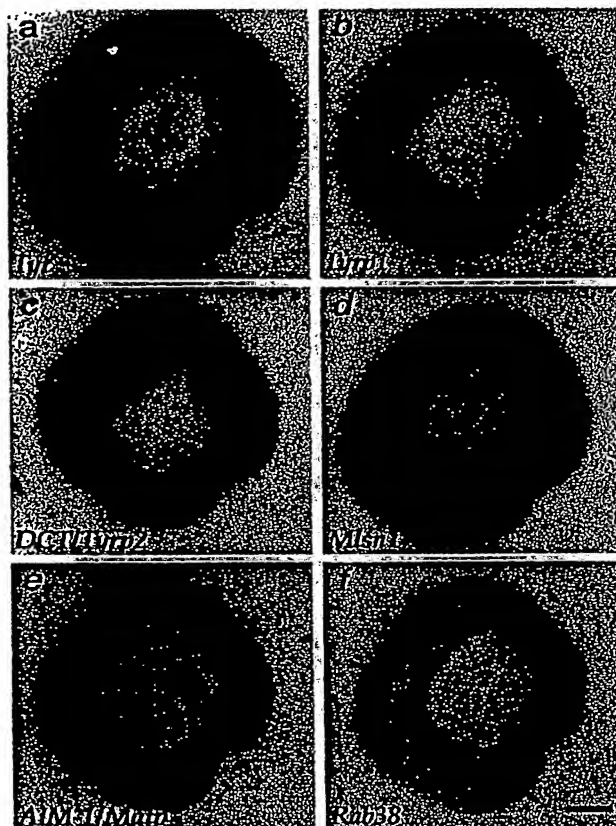


Fig. 2

3/7

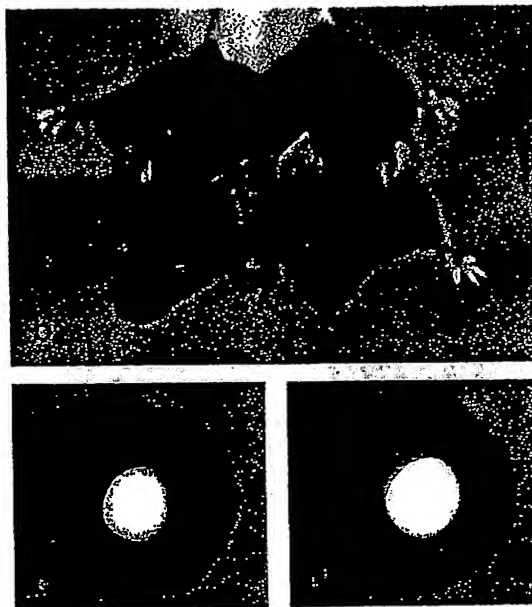
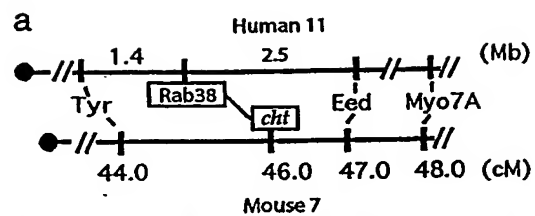


Fig. 3

4/7

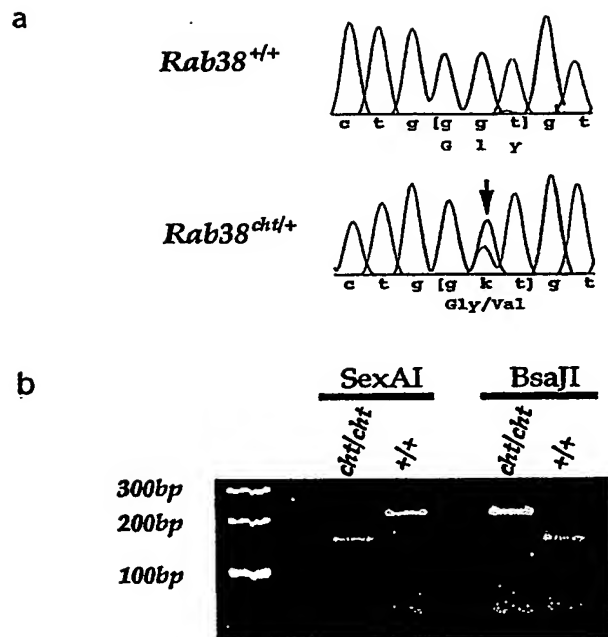


Fig. 4



5/7

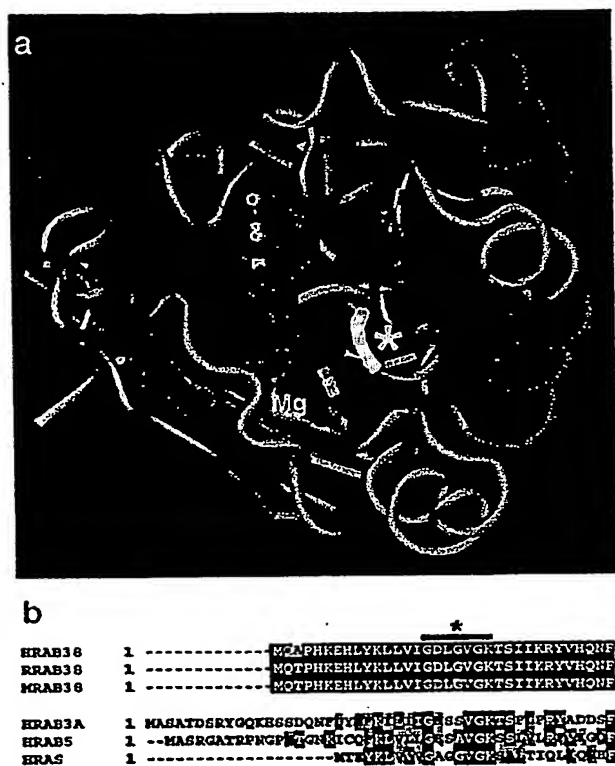


Fig. 5

6/7

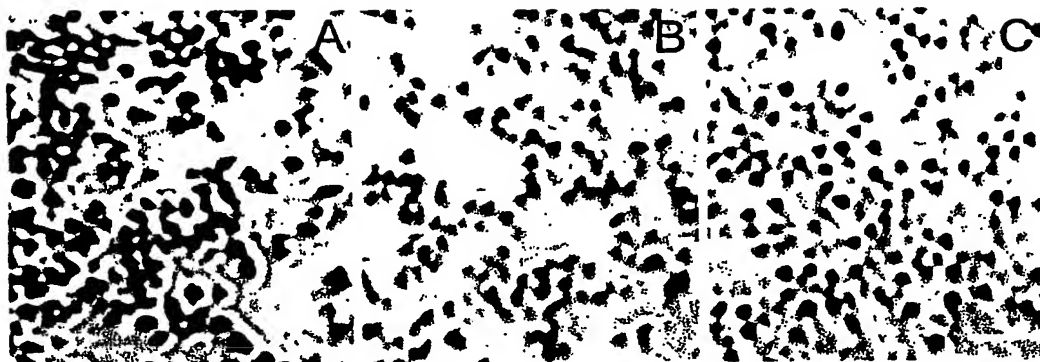


Fig. 6

7/7

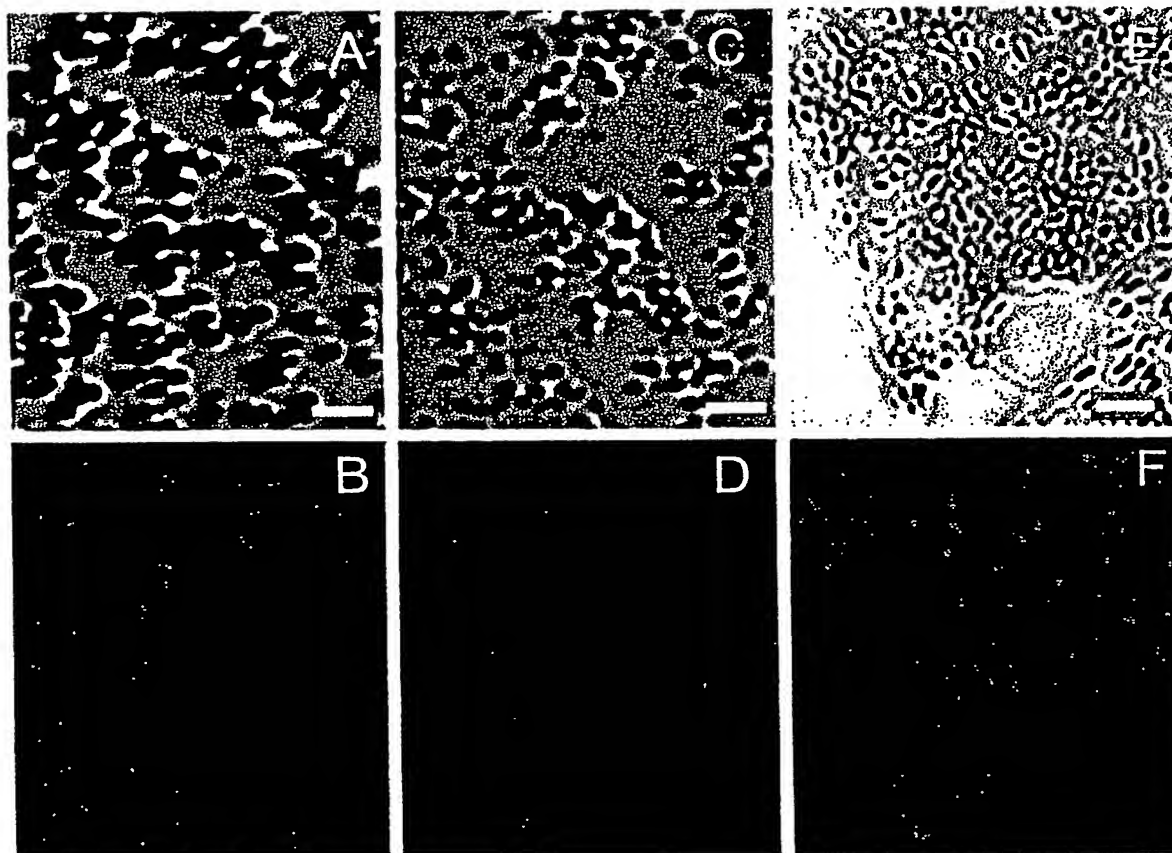


Fig. 7

## SEQUENCE LISTING

&lt;110&gt; Pavan, William J.

Loftus, Stacie K.

&lt;120&gt; Alteration Of RAB38 Function To Modulate Mammalian Pigmentation

&lt;130&gt; NIH-05102

&lt;160&gt; 26

&lt;170&gt; PatentIn version 3.1

&lt;210&gt; 1

&lt;211&gt; 8

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

<400> 1  
ctgggtgt

8

&lt;210&gt; 2

&lt;211&gt; 8

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

<400> 2  
ctgggtgt

8

&lt;210&gt; 3

&lt;211&gt; 34

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 3

Met Gln Ala Pro His Lys Glu His Leu Tyr Lys Leu Leu Val Ile Gly  
1 5 10 15Asp Leu Gly Val Gly Lys Thr Ser Ile Ile Lys Arg Tyr Val His Gln  
20 25 30

Asn Phe

&lt;210&gt; 4

&lt;211&gt; 34

&lt;212&gt; PRT

&lt;213&gt; Rattus norvegicus

&lt;400&gt; 4

Met Gln Thr Pro His Lys Glu His Leu Tyr Lys Leu Leu Val Ile Gly  
 1 5 10 15

Asp Leu Gly Val Gly Lys Thr Ser Ile Ile Lys Arg Tyr Val His Gln  
 20 25 30

Asn Phe

&lt;210&gt; 5

&lt;211&gt; 34

&lt;212&gt; PRT

&lt;213&gt; Mus musculus

&lt;400&gt; 5

Met Gln Ala Pro His Lys Glu His Leu Tyr Lys Leu Leu Val Ile Gly  
 1 5 10 15

Asp Leu Gly Val Gly Lys Thr Ser Ile Ile Lys Arg Tyr Val His Gln  
 20 25 30

Asn Phe

&lt;210&gt; 6

&lt;211&gt; 47

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 6

Met Ala Ser Ala Thr Asp Ser Arg Tyr Gly Gln Lys Glu Ser Ser Asp  
 1 5 10 15

Gln Asn Phe Asp Tyr Met Phe Lys Ile Leu Ile Ile Gly Asn Ser Ser  
 20 25 30

Val Gly Lys Thr Ser Phe Leu Phe Arg Tyr Ala Asp Asp Ser Phe  
           35                          40                          45

<210> 7

<211> 45

<212> PRT

<213> Homo sapiens

<400> 7

Met Ala Ser Arg Gly Ala Thr Arg Pro Asn Gly Pro Asn Thr Gly Asn  
   1                  5                          10                          15

Lys Ile Cys Gln Phe Lys Leu Val Leu Leu Gly Glu Ser Ala Val Gly  
           20                          25                          30

Lys Ser Ser Leu Val Leu Arg Phe Val Lys Gly Gln Phe  
           35                          40                          45

<210> 8

<211> 28

<212> PRT

<213> Homo sapiens

<400> 8

Met Thr Glu Tyr Lys Leu Val Val Val Gly Ala Gly Gly Val Gly Lys  
   1                  5                          10                          15

Ser Ala Leu Thr Ile Gln Leu Ile Gln Asn His Phe  
           20                          25

<210> 9

<211> 1439

<212> DNA

<213> Mus musculus

<220>

<221> misc\_feature

<223> Sequence is mRNA.

<400> 9  
 acatagagct ccgggaaacg tcggtgccca gccccggctg tgcttcccag agcaagctcc 60  
 aggctccgca agaccgcggg gcctccagga tgcagacacc tcacaaggag cacctgtaca 120  
 agctgctggt gatcggcgac ctgggtgtgg gcaagaccag cattatcaag cgctatgtgc 180

```

acaaaaactt ctctctgcac taccggggcca ccattggtgt ggacttcgcg ctgaagggtgc 240
tccactggga cccagagacg gtggtgcgct tgcagctctg ggacattgct ggtcaagaaa 300
gatttgga aa catgacaaga gtttattacc ggggaagctat gggggcattt attgtttttg 360
atgtcaccag accagccaca ttgaagccg tggcaaagt gaaaaatgat ttggactcaa 420
agttaacgct ccctaattggt aagccagtgt cagtgggtct gttggccaac aaatgtgacc 480
aagggaagga tgtgcttatg aacaatggac tcaagatgga ccagttctgc aaggagcatg 540
gcttcgtagg atggtttgaa acatcagcca aggaaaacat aaacattgat gaagcctcaa 600
gatgcctggt caagcacata cttgcaaatg agtgtgacct cctagagtct atagaaccgg 660
acattgtgaa gccccatctc acatcgccca aggttgtcag ctgctctggc tgtgccaaat 720
cctagaaggc tcctctgctg gcatatgaca gacagaacct gtggccctca tgaatcgtgc 780
ttcagttttt ccttattacc attttgggta agcgtcagga tagggaagca catgtgacaa 840
gccaaagata catgactgta tggttcctgt caaagaggaa cagcaaatgt tctttatgtg 900
ttttcccacc ccatcagcac agtgtttaca agcttttaaa atattagtct gtcacaatat 960
gctgttttat cattgagcaa agccactcag ggacacagac agccctaata tttgttcctt 1020
taaatacaaa aaggcttctg gtcttcttga gaaggggaat aacagagcaa ggcagagggtc 1080
aagctaagtg tggggatttg tcttgccctg gtgtgtcttt gttcaggtat caatttggtc 1140
ccgggtgggtc tgataggtct attaaataga aaccattcat ggtagacctt agggttgkct 1200
gtgatgtttc tcttcagagt cgtgtgcaca ggcagcctgg gcttttgttg tcaattgctg 1260
tgccctgaat gctggtttaa ctgaaaactg tatggaaaga tctgctccct gtatgtgctt 1320
ttctttcagc ttctctgac tcaagctgca ggactottct gtatgtggaa gatataattat 1380
atatatTTTT cacaagtga aaataaaaaca ttaaaaatgc tgtttccctg tttctgata 1439

```

&lt;210&gt; 10

&lt;211&gt; 291

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

```

<400> 10
acatagagct ccgggaaacg tcggtgcca gccccggctg tgcttcccag agcaagctcc 60
aggctccgca agaccgcggg gcctccagga tgcagacacc tcacaaggag cacctgtaca 120
agctgctggt gatcggcgac ctgggtgtgg gcaagaccag cattatcaag cgctatgtgc 180
acaaaaactt ctctctgcac taccggggcca ccattggtgt ggacttcgcg ctgaagggtgc 240
tccactggga cccagagacg gtggtgcgct tgcagctctg ggacattgct g 291

```

&lt;210&gt; 11

&lt;211&gt; 281

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 11

```

gtcaagaaag atttggaac atgacaagag ttattaccg ggaagctatg ggggcattta      60
ttgtttttga tgtcaccaga ccagccacat ttgaagccgt ggcaaagtgg aaaaatgatt    120
tggaactcaa gttaacgctc cctaattgga agccagtgtc agtggttctg ttggccaaca    180
aatgtgacca agggaaggat gtgcttatga acaatggact caagatggac cagttctgca    240
aggagcatgg ctctcgttaga tggtttgaaa catcagccaa g                        281

```

&lt;210&gt; 12

&lt;211&gt; 868

&lt;212&gt; DNA

&lt;213&gt; Mus musculus

&lt;400&gt; 12

```

ggaaaacata aacattgatg aagcctcaag atgcctggtc aagcacatac ttgcaaata      60
gtgtgacctc ctagagtcta tagaaccgga cattgtgaag ccccatctca catcgcccaa    120
ggttgtcagc tgctctggct gtgccaaatc ctagaaggct cctctgctgg catatgacag    180
acagaacccg tggccctcat gaatcgtgct tcagtttttc cttattacca ttttgggtaa    240
gcgtcaggat agggaagcac atgtgacaag ccaaagatac atgactgtat ggttcctgtc    300
aaagaggaac agcaaatggt ctttatgtgt tttcccaccc catcagcaca gtgtttacaa    360
gcttttaaaa tattagtctg tcacaatatg ctgtttttatc attgagcaaa gccactcagg    420
gacacagaca gccctaatat ttgttccttt aaatcaacaa aggcttctgg tcttcttgag    480
aaggggaata acagagcaag gcagagggtc agctaagtgt ggggatttgt cttgccctgg    540
tgtgtctttg ttcaggatc aatttggtcc cgggtggtct gataggctc ttaaataga      600
accattcatg gtagacctaa gggttgkctg tgatgtttct cttcagagtc gtgtgcacag    660
gcagcctggg cttttgttgt cacttgctgt gccctgaatg ctggtttaac tgaaaactgt    720
atggaaagat ctgctccctg tatgtgcctt tctttcagct tcctctgact caagctgcag    780
gactcttctg tatgtggaag atatattata tatatttttc acaagtgaag aataaaacat    840
taaaaatgct gtttcctgt ttctgata                                     868

```



<210> 13

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 13

gcgcgaatta accctcacta aagggtctga gcacccctgt cttct

45

<210> 14

<211> 45

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 14

gcgcgtaata cgactcacta tagggcccag ttgcaaaatt ccagt

45

<210> 15

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 15

gcgggtaata cgactcacta taggggccac aaacatgtcc tacttac

47

<210> 16

<211> 44

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 16

gcgcgaatta accctcacta aagggaagct tccggactct ctac

44

<210> 17  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 17  
taggaaggag gattaaaccc g 21  
<210> 18  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 18  
gaactcctca tggctcactc c 21  
<210> 19  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 19  
ggatatgaag ctccagtgtgta gtgtac 26  
<210> 20  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 20  
cactggacag aaacattatt gtcac 25

<210> 21  
<211> 26  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 21  
aagttatcag ccagtgagat actgtg 26  
<210> 22  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 22  
cacatgtggg atatctatcc tgacg 25  
<210> 23  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 23  
ggcctccagg atgcagacac c 21  
<210> 24  
<211> 21  
<212> DNA  
<213> Artificial Sequence  
<220>  
<223> Synthetic  
<400> 24  
ccagcaatgt cccagagctg c 21

<210> 25

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 25

ggggacaagt ttgtacaaaa aagcaggctc catgcagaca cctcacaag

49

<210> 26

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 26

ggggaccact ttgtacaaga aagctggggt ctaggatttg gcacagccag a

51

<210> 25

<211> 49

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 25

ggggacaagt ttgtacaaaa aagcaggctc catgcagaca cctcacaag

49

<210> 26

<211> 51

<212> DNA

<213> Artificial Sequence

<220>

<223> Synthetic

<400> 26

ggggaccact ttgtacaaga aagctggggt ctaggatttg gcacagccag a

51

# Human RAB38 DNA sequence

```

1 ggctgcgctt ccctggctcag gcacggcacg tctggccggc cgccaggatg caggccccgc
61 acaaggagca cctgtacaag ttgctgggtga ttggcgacct gggcgtgggg aagaccagta
121 tcatcaagcg ctacgtgcac cagaacttct cctcgacta cggggccaca atcggcgtgg
181 acttcgcgct caaggtgctc cactgggacc cggagactgt ggtgcgcctg cagctctggg
241 atatcgcagg tcaagaaaga ttggaaaca tgacgagggt ctattaccga gaagctatgg
301 gtgcatttat tgtcttcgat gtcaccaggc cagccacatt tgaagcagtg gcaaagtggg
361 aaaatgattt ggactccaag ttaagtctcc ctaatggcaa accggtttca gtggttttgt
421 tggccaacaa atgtgaccag ggggaaggatg tgctcatgaa caatggcctc aagatggacc
481 agttctgcaa ggagcacggt ttcgtaggat ggtttgaaac atcagcaaag gaaaatataa

```

```
541 acattgatga agcctccaga tgcctgggta aacacatact tgcaaatgag tgtgacctaa
601 tggagttctat tgagccggac gtcgtgaagc cccatctcac atcaaccaag gttgccagct
661 gctctgggctg tgccaaatcc tagtaggcac ctttgctggg gtctggtagg aatgacctca
721 ttgttccaca aattgtgcct ctatttttac cattttgggt aaacgtcagg atagatatac
781 cacatgtggc aagccaaaga tctatgcctc tgtttttca atgagagaga aatagcaa
841 gttctttcta tgctttcctc accatcatca cagtgtttac aaacttttga aaatatttag
901 tctgttaciaa acttctgtca tgtagctgac caaaatcctg cagggccaca gtcggcactg
961 ttattttgctt cttttaatca gcaaaggcct caagtcttaa aataaaaggg gagaagaaca
1021 aactagctgt caagtcaagg actggctttc accttgccct ggtgtctttt tccagatttc
1081 aatatattct ctgatggcct gacaggccta ttaagtagat gtgatatttt cttccaagat
1141 gacctccatt ctcggcagac ctaagagttg cctctgagtt agctctttgg aatcgtgaac
1201 acaggtgtgc tatattgtcc ttgtcctaac tgtcacttgc catggcctga atgttggtt
1261 aactgaatat tgtatgaaaa gacatgcctc catatgtgcc tttctgttag ctctctttga
1321 ctcaagctgt ggggctcctc tatacatgct atacatgtaa tatatattat atatattttt
1381 gcaagtgaac aataaaacat taaaagataa aa
```

SF 1423179 v1

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**